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## (54) Title: VIABLE VIRUSES WITH FOREIGN TAGS

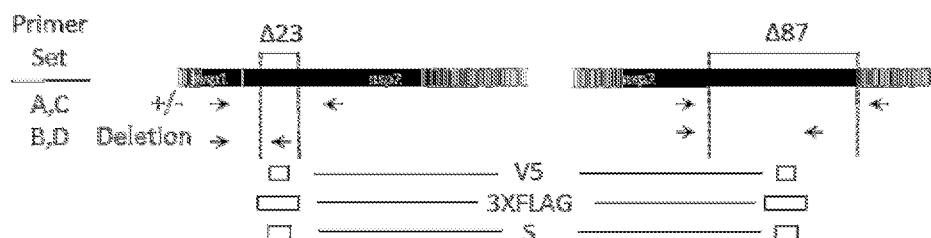


FIG. 1A

(57) Abstract: Multiple DIVA vaccines effective against porcine reproductive and respiratory syndrome virus (PRRSV) are disclosed. The DIVA vaccines may be negative DIVAs or positive DIVAs. The DIVA vaccines may be produced by modifying the nsp2 region of a modified live virus vaccine. The modification may be one or more deletions only (negative DIVAs) or a deletion with an insertion (positive DIVAs). The insertion may be of an epitope tag, such as a V5, S-Tag, or FLAG tag. Produced DIVA vaccines may be stable through multiple passes and thus may be effective for production and vaccination of animals.



## VIABLE VIRUSES WITH FOREIGN TAGS

### BACKGROUND

[0001] Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the viral family *Arteriviridae*, causes respiratory disease in growing swine and fetal mortality. Efforts to contain this virus have included early diagnosis, enhanced biosecurity measures, herd management and application of vaccines (Holtkamp et al., 2013. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *Journal of Swine Health and Production* 21, 72-84; Pileri and Mateu, 2016. Review on the transmission porcine reproductive and respiratory syndrome virus between pigs and farms and impact on vaccination. *Vet. Res.* 47, 108). However, the available modified live vaccines (MLV) only provide partial protection against heterologous viruses and are dwarfed by the number of PRRSV strains circulating worldwide. PRRSV strains vary greatly and each exists as one of two genetically different types (Type 1 and 2). Each genotype continues to evolve by both imprecise replication due to its inherent polymerase error rate and by recombination (Hanada et al., 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. *Mol. Biol. Evol.* 22, 1024-1031; Kappes and Faaborg, 2015. PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. *Virology* 479-480, 475-486; Shi et al., 2013. Recombination is associated with an outbreak of novel highly pathogenic porcine reproductive and respiratory syndrome viruses in China. *J. Virol.* 87, 10904-10907; Shi et al., 2010. Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Res.* 154, 7-17).

The swine industry is also hampered by the lack of a vaccine that effectively differentiates infected from vaccinated animals (DIVA).

**[0002]** In PRRSV infected cells, the nsp2 protein not only is present as a full-length protein but is processed into variably sized isomers (Wang, et al., 2008. Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence. *Virology* 371, 418-429; Yuan, et al., 2000. Heteroclitite subgenomic RNAs are produced in porcine reproductive and respiratory syndrome virus infection. *Virology* 275, 158-169; Yuan, et al., 2004. Characterization of heteroclitite subgenomic RNAs associated with PRRSV infection. *Virus Res.* 105, 75-87.

**[0003]** Ingelvac PRRS® MLV (MLV) is used widely in several countries to dampen the effect of PRRSV herd infection in young pigs, has been used safely for 20 years with few instances of reversion to virulence, replicates sufficiently in vaccinated swine and is a low cost platform that is affordable for the industry (Botner et al., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet. Rec.* 141, 497-499; Jeong et al., 2016. Evaluation of a 20 year old porcine reproductive and respiratory syndrome (PRRS) modified live vaccine (Ingelvac((R)) PRRS MLV) against two recent type 2 PRRS virus isolates in South Korea. *Vet. Microbiol.* 192, 102-109; Martinez-Lobo et al., 2013. Safety of Porcine Reproductive and Respiratory Syndrome Modified Live Virus (MLV) vaccine strains in a young pig infection model. *Vet. Res.* 44, 115; Mengeling et al., 1999. Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. *Am. J. Vet. Res.* 60, 334-340; Nielsen et al., 2001. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J. Gen. Virol.* 82, 1263-1272). Type 2 strain VR-2332, the parent of MLV, causes a mild disease under

defined conditions, thus concerns of simple reversion to virulence are reduced. Moreover, VR-2332 replicates at a lower rate in swine and does not result in a reduction in weight gain when compared to more pathogenic isolates (Guo et al., 2013. Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. *Virology* 435, 372-384; Guo et al., 2013. Chinese and Vietnamese strains of HP-PRRSV cause different pathogenic outcomes in United States high health swine. *Virology* 446, 238-250).

**[0004]** All of the references cited herein, including U.S. Patents and U.S. Patent Application Publications, are incorporated by reference in their entirety.

## **SUMMARY**

**[0005]** According to at least one aspect of the invention, an immunogenic composition may include an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region, wherein the nsp2 region has a modification at one of amino acids 10-14, the modification includes a deletion, and the immunogenic composition is capable of inducing an immune response in a recipient.

**[0006]** According to a further aspect of the invention, the immunogenic composition may also include a pharmaceutically acceptable adjuvant.

**[0007]** According to a further aspect of the invention, the immunogenic composition may also include a pharmaceutically acceptable carrier.

**[0008]** According to a further aspect of the invention, the modification may be at amino acid 12.

**[0009]** According to a further aspect of the invention, the amino acid sequence of the nsp2 region may be one of a sequence of SEQ ID NO: 3 and a sequence having at least 95% homology to SEQ ID NO: 3.

**[0010]** According to a further aspect of the invention, the modification may further include an insertion, and the insertion includes the insertion of an epitope tag of 10-25 amino acids in length.

**[0011]** According to a further aspect of the invention, the epitope tag may be one of a V5 tag, an S-Tag, and a FLAG tag.

**[0012]** According to a further aspect of the invention, the amino acid sequence of the nsp2 region may be one of a sequence of SEQ ID NO: 13, a sequence of SEQ ID NO: 15, a sequence of SEQ ID NO: 17, a sequence having at least 95% homology to SEQ ID NO: 13, a sequence having at least 95% homology to SEQ ID NO: 15, and a sequence having at least 95% homology to SEQ ID NO: 17.

**[0013]** According to another aspect of the invention, an immunogenic composition may include an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region, wherein the nsp2 region has a modification at amino acid 724-728, the modification includes a deletion, and the immunogenic composition is capable of inducing an immune response in a recipient.

**[0014]** According to a further aspect of the invention, the modification may be at amino acid 726.

**[0015]** According to a further aspect of the invention, the amino acid sequence of the nsp2 region may be one of a sequence of SEQ ID NO: 5 and a sequence having at least 95% homology to SEQ ID NO: 5.

**[0016]** According to a further aspect of the invention, the modification may further include an insertion, and the insertion may include the insertion of an epitope tag of 10-25 amino acids in length.

**[0017]** According to a further aspect of the invention, the epitope tag may be one of a V5 tag, an S-Tag, and a FLAG tag.

**[0018]** According to a further aspect of the invention, the amino acid sequence of the nsp2 region may be one of a sequence of SEQ ID NO: 19, a sequence of SEQ ID NO: 21, a sequence of SEQ ID NO: 23, a sequence having at least 95% homology to SEQ ID NO: 19, a sequence having at least 95% homology to SEQ ID NO: 21, and a sequence having at least 95% homology to SEQ ID NO: 23.

**[0019]** According to another aspect of the invention, an immunogenic composition may include an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region, wherein the nsp2 region includes a deletion at a deletion starting point, the deletion starting point being one of amino acids 10-14 or one of amino acids 724-728, the deletion including the removal of 21-89 amino acids, and the nsp2 region optionally includes an insertion of an epitope tag of 10-25 amino acids in length at the deletion starting point.

**[0020]** According to another aspect of the invention, a method for reducing the incidence of porcine reproductive and respiratory syndrome virus (PRRSV) in swine may include administering to a swine an immunogenic composition in an amount effective to generate an immune response in said swine to said PRRSV, wherein the immunogenic composition includes an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region, the nsp2 region has a

modification at one of amino acids 10-14 or one of amino acids 724-728, and the modification includes a deletion.

**[0021]** According to a further aspect of the invention, the immune response may be protection against porcine reproductive and respiratory syndrome virus.

**[0022]** According to a further aspect of the invention, the modification further includes an insertion, and the insertion includes the insertion of an epitope tag of 10-25 amino acids in length.

## SEQUENCE LISTING

**[0023]** The Sequence Listing submitted via EFS-Web as an ASCII compliant text file format (.txt) filed May 30, 2017, named “SequenceListing-013316\_ST25” (created on May 30, 2017, 126 kb), is hereby incorporated herein by reference in its entirety. This Sequence Listing serves as paper copy of the Sequence Listing required by 37 C.F.R. § 1.821(c) and the Sequence Listing in computer-readable form (CRF) required by 37 C.F.R. § 1.821(e). A statement under 37 C.F.R. § 1.821(f) is not necessary.

**[0024]** The sequences used or referenced to in the present invention are described in Table 1:

Table 1: Sequence Descriptions

|              |                                                                     |
|--------------|---------------------------------------------------------------------|
| SEQ ID NO: 1 | cDNA sequence encoding nsp2 region of pMLV                          |
| SEQ ID NO: 2 | cDNA sequence encoding nsp2 region of nsp2 $\Delta$ 23 DIVA vaccine |
| SEQ ID NO: 3 | Amino acid sequence of nsp2 region of nsp2 $\Delta$ 23 DIVA vaccine |
| SEQ ID NO: 4 | cDNA sequence encoding nsp2 region of nsp2 $\Delta$ 87 DIVA vaccine |
| SEQ ID NO: 5 | Amino acid sequence of nsp2 region of nsp2 $\Delta$ 87 DIVA vaccine |
| SEQ ID NO: 6 | cDNA sequence encoding V5 tag                                       |
| SEQ ID NO: 7 | Amino acid sequence of V5 tag                                       |
| SEQ ID NO: 8 | cDNA sequence encoding S-Tag tag                                    |

|               |                                                                            |
|---------------|----------------------------------------------------------------------------|
| SEQ ID NO: 9  | Amino acid sequence of S-Tag tag                                           |
| SEQ ID NO: 10 | cDNA sequence encoding FLAG tag                                            |
| SEQ ID NO: 11 | Amino acid sequence of FLAG tag                                            |
| SEQ ID NO: 12 | cDNA sequence encoding nsp2 region of nsp2Δ23-V5 DIVA vaccine              |
| SEQ ID NO: 13 | Amino acid sequence of nsp2 region of nsp2Δ23-V5 DIVA vaccine              |
| SEQ ID NO: 14 | cDNA sequence encoding nsp2 region of nsp2Δ23- S-Tag DIVA vaccine          |
| SEQ ID NO: 15 | Amino acid sequence of nsp2 region of nsp2Δ23- S-Tag DIVA vaccine          |
| SEQ ID NO: 16 | cDNA sequence encoding nsp2 region of nsp2Δ23-FLAG DIVA vaccine            |
| SEQ ID NO: 17 | Amino acid sequence of nsp2 region of nsp2Δ23- FLAG DIVA vaccine           |
| SEQ ID NO: 18 | cDNA sequence encoding nsp2 region of nsp2Δ87-V5 DIVA vaccine              |
| SEQ ID NO: 19 | Amino acid sequence of nsp2 region of nsp2Δ87-V5 DIVA vaccine              |
| SEQ ID NO: 20 | cDNA sequence encoding nsp2 region of nsp2Δ87- S-Tag DIVA vaccine          |
| SEQ ID NO: 21 | Amino acid sequence of nsp2 region of nsp2Δ87- S-Tag DIVA vaccine          |
| SEQ ID NO: 22 | cDNA sequence encoding nsp2 region of nsp2Δ87-FLAG DIVA vaccine            |
| SEQ ID NO: 23 | Amino acid sequence of nsp2 region of nsp2Δ87- FLAG DIVA vaccine           |
| SEQ ID NO: 24 | cloning vector designated “pCHAZ” (circular)                               |
| SEQ ID NO: 25 | Primer A1 used to verify non-contamination                                 |
| SEQ ID NO: 26 | Primer A2 used to verify non-contamination                                 |
| SEQ ID NO: 27 | Primer B1 used to verify non-contamination                                 |
| SEQ ID NO: 28 | Primer B2 used to verify non-contamination                                 |
| SEQ ID NO: 29 | Primer C1 used to verify non-contamination                                 |
| SEQ ID NO: 30 | Primer C2 used to verify non-contamination                                 |
| SEQ ID NO: 31 | Primer D1 used to verify non-contamination                                 |
| SEQ ID NO: 32 | Primer D2 used to verify non-contamination                                 |
| SEQ ID NO: 33 | Oligonucleotide probe used in Northern blot analysis for negative DIVAs    |
| SEQ ID NO: 34 | Oligonucleotide probe used in Northern blot analysis for V5-tagged DIVAs   |
| SEQ ID NO: 35 | Oligonucleotide probe used in Northern blot analysis for FLAG-tagged DIVAs |
| SEQ ID NO: 36 | Oligonucleotide probe used in Northern blot analysis for S-tagged DIVAs    |

## BRIEF DESCRIPTION OF THE FIGURES

[0025] Advantages of embodiments of the present invention will be apparent from the following detailed description of the exemplary embodiments. The following detailed description should be considered in conjunction with the accompanying figures in which:

[0026] Exemplary FIG. 1A shows a schematic representation of two negative DIVAs ( $\Delta 23$  and  $\Delta 87$ ), three potential insertion flags (V5, FLAG, and S), and primer sets which can be used to confirm no contamination is present.

[0027] Exemplary FIG. 1B shows the results of RT-PCR analysis to determine no contamination was present.

[0028] Exemplary FIG. 1C shows the results of restriction enzyme analysis to determine no contamination was present.

[0029] Exemplary FIG. 2 shows a schematic of each DIVA vaccine as produced and after 3 and 10 passages, showing the locations of any mutations found.

[0030] Exemplary FIG. 3 shows Northern blot analysis to determine the amount and/or effect of any subgenomic RNA (sgRNA).

[0031] Exemplary FIG. 4A shows data for the growth rate of some DIVA vaccines.

[0032] Exemplary FIG. 4B shows images of plaque growth at a third passage for some DIVA vaccines.

[0033] Exemplary FIG. 4C shows an immunofluorescence assay in MARC-145 cells to determine localization, if any, of some DIVA vaccines in a cellular system.

[0034] Exemplary FIG. 4D shows an immunofluorescence assay in MARC-145 cells with a co-infection of  $\Delta 23$ -S and  $\Delta 87$ -V5 tagged DIVAs, revealing some differentiation in non-perinuclear localization.

## DETAILED DESCRIPTION

**[0035]** Aspects of the invention are disclosed in the following description and related drawings directed to specific embodiments of the invention. Alternate embodiments may be devised without departing from the spirit or the scope of the invention. Additionally, well-known elements of exemplary embodiments of the invention will not be described in detail or will be omitted so as not to obscure the relevant details of the invention. Further, to facilitate an understanding of the description discussion of several terms used herein follows.

**[0036]** As used herein, the word “exemplary” means “serving as an example, instance or illustration.” The embodiments described herein are not limiting, but rather are exemplary only. It should be understood that the described embodiment are not necessarily to be construed as preferred or advantageous over other embodiments. Moreover, the terms “embodiments of the invention”, “embodiments” or “invention” do not require that all embodiments of the invention include the discussed feature, advantage or mode of operation.

**[0037]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. As used herein, the term “about” refers to a quantity, level, value, or amount that varies by as much as 30%, preferably by as much as 20%, and more preferably by as much as 10% to a reference quantity, level, value, or amount. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

**[0038]** The amounts, percentages, and ranges disclosed herein are not meant to be limiting, and increments between the recited amounts, percentages, and ranges are specifically envisioned as part of the invention.

**[0039]** “Optional” or “optionally” means that the subsequently described event or circumstance may or may not occur, and that the description includes instances in which said event or circumstance occurs and instances where it does not. For example, the phrase “optionally comprising X” means that the composition may or may not contain X, and that this description includes compositions that contain and do not contain X.

**[0040]** Because this invention involves production of genetically altered viruses and involves recombinant DNA techniques, the following definitions are provided to assist in describing this invention. The terms “isolated”, “purified”, or “biologically pure” as used herein, refer to material that is substantially or essentially free from components that normally accompany the material in its native state or when the material is produced. In an exemplary embodiment, purity and homogeneity are determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A nucleic acid or particular bacteria that are the predominant species present in a preparation is substantially purified. In an exemplary embodiment, the term “purified” denotes that a nucleic acid or protein that gives rise to essentially one band in an electrophoretic gel. Typically, isolated nucleic acids or proteins have a level of purity expressed as a range. The lower end of the range of purity for the component is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

**[0041]** For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp), or nucleotides (nt). Estimates are typically derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kiloDaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel

electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

**[0042]** Unless otherwise indicated, a particular nucleic acid sequence for each amino acid substitution (alteration) also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), the complementary (or complement) sequence, and the reverse complement sequence, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (*see e.g.*, Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98(1994)). Because of the degeneracy of nucleic acid codons, one can use various different polynucleotides to encode identical polypeptides. Table 2, *infra*, contains information about which nucleic acid codons encode which amino acids and is useful for determining the possible nucleotide substitutions that are included in this invention.

Table 2

| Amino acid | Nucleic acid codons          | Amino acid | Nucleic acid codons          |
|------------|------------------------------|------------|------------------------------|
| Ala/A      | GCT, GCC, GCA, GCG           | Leu/L      | TTA, TTG, CTT, CTC, CTA, CTG |
| Arg/R      | CGT, CGC, CGA, CGG, AGA, AGG | Lys/K      | AAA, AAG                     |
| Asn/N      | AAT, AAC                     | Met/M      | ATG                          |
| Asp/D      | GAT, GAC                     | Phe/F      | TTT, TTC                     |
| Cys/C      | TGT, TGC                     | Pro/P      | CCT, CCC, CCA, CCG           |
| Gln/Q      | CAA, CAG                     | Ser/S      | TCT, TCC, TCA, TCG, AGT, AGC |
| Glu/E      | GAA, GAG                     | Thr/T      | ACT, ACC, ACA, ACG           |
| Gly/G      | GGT, GGC, GGA, GGG           | Trp/W      | TGG                          |
| His/H      | CAT, CAC                     | Tyr/Y      | TAT, TAC                     |
| Ile/I      | ATT, ATC, ATA                | Val/V      | GTT, GTC, GTA, GTG           |
| Stop       | TAA, TGA, TAG                |            |                              |

[0043] The term “primer” refers to an oligonucleotide, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. A primer may occur naturally, as in a purified restriction digest, or may be produced synthetically.

[0044] A primer is selected to be “substantially complementary” to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence is sufficiently complementary with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

[0045] Oligonucleotides and polynucleotides that are not commercially available can be chemically synthesized *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage and Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), or using an automated synthesizer, as described in Van Devanter et al., *Nucleic Acids Res.* 12:6159-6168 (1984). Other methods for synthesizing oligonucleotides and polynucleotides are known in the art. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

[0046] The terms “identical” or percent “identity”, in the context of two or more polynucleotides or polypeptide sequences, refer to two or more sequences or sub-sequences that are the same or have a specified percentage of nucleotides or amino acids (respectively) that are the same (*e.g.*,

80%, 85% identity, 90% identity, 99%, or 100% identity), when compared and aligned for maximum correspondence over a designated region as measured using a sequence comparison algorithm or by manual alignment and visual inspection.

**[0047]** The phrase “high percent identical” or “high percent identity”, in the context of two polynucleotides or polypeptides, refers to two or more sequences or sub-sequences that have at least about 80% identity, at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% nucleotide or amino acid identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. In an exemplary embodiment, a high percent identity exists over a region of the sequences that is at least about 16 nucleotides or amino acids in length. In another exemplary embodiment, a high percent identity exists over a region of the sequences that is at least about 50 nucleotides or amino acids in length. In still another exemplary embodiment, a high percent identity exists over a region of the sequences that is at least about 100 nucleotides or amino acids or more in length. In one exemplary embodiment, the sequences are high percent identical over the entire length of the polynucleotide or polypeptide sequences.

**[0048]** For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. Methods of alignment of sequences for comparison

are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of various algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Ausubel et al. (eds.), Current Protocols in Molecular Biology, 1995 supplement).

**[0049]** The “complement” of a particular polynucleotide sequence is that nucleotide sequence which would be capable of forming a double-stranded DNA or RNA molecule with the represented nucleotide sequence, and which can be derived from the represented nucleotide sequence by replacing the nucleotides by their complementary nucleotide according to Chargaff’s rules ( $A \leftrightarrow T$ ;  $G \leftrightarrow C$ ) and reading in the 5’ to 3’ direction, i.e., in opposite direction of the represented nucleotide sequence (reverse complement).

**[0050]** As used herein, the term “promoter” refers to a polynucleotide that, in its native state, is located upstream or 5' to a translational start codon of an open reading frame (or protein-coding region) and that is involved in recognition and binding of RNA polymerase and other proteins (trans-acting transcription factors) to initiate transcription.. The promoters that are predominately functional in a specific tissue or set of tissues are considered “tissue-specific promoters”. A promoter can be used as a 5' regulatory element for modulating expression of a particular desired polynucleotide (heterologous polynucleotide) operably linked thereto. When operably linked to a transcribable polynucleotide, a promoter typically causes the transcribable polynucleotide to be transcribed in a manner that is similar to that of which the promoter is normally associated.

**[0051]** The term “vector” refers to DNA, RNA, a protein, or polypeptide that are to be introduced into a host cell or organism. The polynucleotides, protein, and polypeptide which are to be introduced into a host can be therapeutic or prophylactic in nature; can encode or be an antigen; can be regulatory in nature; etc. There are various types of vectors including viruses, viroids, plasmids, bacteriophages, cosmids, and bacteria.

**[0052]** An expression vector is nucleic acid capable of replicating in a selected host cell or organism. An expression vector can replicate as an autonomous structure, or alternatively can integrate, in whole or in part, into the host cell chromosomes or the nucleic acids of an organelle, or it is used as a shuttle for delivering foreign DNA to cells, and thus replicate along with the host cell genome. Thus, an expression vector are polynucleotides capable of replicating in a selected host cell, organelle, or organism, e.g., a plasmid, virus, artificial chromosome, nucleic acid fragment, and for which certain genes on the expression vector (including genes of interest) are transcribed and translated into a polypeptide or protein within the cell, organelle or organism; or any suitable construct known in the art, which comprises an “expression cassette”. In contrast, as described in the examples herein, a “cassette” is a polynucleotide containing a section of an expression vector. The use of the cassettes assists in the assembly of the expression vectors. An expression vector is a replicon, such as plasmid, phage, virus, chimeric virus, or cosmid, and which contains the desired polynucleotide sequence operably linked to the expression control sequence(s).

**[0053]** A heterologous polynucleotide sequence is operably linked to one or more transcription regulatory elements (*e.g.*, promoter, terminator and, optionally, enhancer) such that the transcription regulatory elements control and regulate the transcription and/or translation of that heterologous polynucleotide sequence. A cassette can have the heterologous polynucleotide

operably linked to one or more transcription regulatory elements. As used herein, the term “operably linked” refers to a first polynucleotide, such as a promoter, connected with a second transcribable polynucleotide, such as a gene of interest, where the polynucleotides are arranged such that the first polynucleotide affects the transcription of the second polynucleotide. In some embodiments, the two polynucleotide molecules are part of a single contiguous polynucleotide. In other embodiments, the two polynucleotides are adjacent. For example, a promoter is operably linked to a gene of interest if the promoter regulates or mediates transcription of the gene of interest in a cell. Similarly, a terminator is operably linked to the polynucleotide of interest if the terminator regulates or mediates transcription of the polynucleotide of interest, and in particular, the termination of transcription. Constructs of the present invention would typically contain a promoter operably linked to a transcribable polynucleotide operably linked to a terminator.

**[0054]** The term “recombinant” when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, organism, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant viruses may express genes/polynucleotides that are not found within the native (non-recombinant or wild-type) form of the viruses or express native genes in an otherwise abnormal amount – over-expressed, under-expressed or not expressed at all – compared to the non-recombinant or wild-type sequence, polynucleotide, or organism.

**[0055]** A “vaccine” is defined herein as a biological agent which is capable of providing a protective response in an animal to which the vaccine has been delivered and is incapable of causing severe disease. Administration of the vaccine results in increased immunity from a disease; the vaccine stimulates antibody production or cellular immunity against the pathogen

causing the disease. Immunity is defined herein as the induction of a significantly higher level of protection in a population of recipients, such as porcine, against mortality and clinical symptoms after vaccination compared to an unvaccinated group. In particular, the vaccine(s) according to the invention can: (a) protect a large proportion of vaccinated animals against the occurrence of clinical symptoms of the disease and mortality, or; (b) result in a significant decrease in clinical symptoms of the disease and mortality.

**[0056]** The term “effective amount” of a compound or property as provided herein is meant such amount as is capable of performing the function of the compound or property for which an effective amount is expressed. As will be pointed out below, the exact amount required will vary from process to process, depending on recognized variables such as the compounds employed and the processing conditions observed. Thus, it is not possible to specify an exact “effective amount.” However, an appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation.

**[0057]** The term “DIVA” or “DIVA vaccine” refers to a vaccine which may be used to differentiate infected from vaccinated animals. Differentiation may be made according to any known method in the art which can substantially identify a DIVA-vaccinated organism from an infected organism. A DIVA vaccine may be, for example, a negative DIVA or a positive DIVA. A negative DIVA vaccine is a vaccine which has been modified by a deletion in one or more regions of its sequence relative to the corresponding region(s) of the virus. A positive DIVA vaccine is a vaccine which has been modified by an insertion in one or more regions of its sequence relative to the corresponding region(s) of the virus. The insertion may be a tag, such as an epitope tag, which may be used to positively identify the vaccine.

**[0058]** The deletions and/or insertions in a DIVA vaccine may vary in length. Deletions according to the present invention may be as small as 10 amino acids in length or as large as 120 amino acids in length (and the corresponding size in the encoding cDNA sequence(s)). Insertions may be as small as 8 amino acids in length or as large as 30 amino acids in length.

**[0059]** In some instances, vaccines of the present invention may also be administered with one or more adjuvants, which includes any material included in the vaccine formulation that enhances an immune response in the recipient that is induced by the vaccine.

**[0060]** An immunogenic composition may refer to a vaccine, optionally including one or more adjuvants, carriers, or other pharmaceutically acceptable materials. Immunogenic compositions may also be referred to as vaccine compositions.

**[0061]** Immunogenic compositions of the present invention can also comprise pharmaceutically or veterinarily acceptable carriers in addition to the recombinant protein component of the vaccine. Carriers utilized in practicing the vaccines provided herein can be any known in the art and can be liquid, solid, semi-solid, or gel. The type of formulation can be modified depending on the route of administration of the antigen. For example, if the vaccines of the present invention are applied parenterally (intramuscularly, intravascularly, or subcutaneously), a liquid formulation – such as an emulsion, suspension, or solution – may be preferred. For oral administration, the vaccines of the present invention can be applied to carriers such as pellets, tablets, kibbles, chewables, powders and beads, as well as specific materials such as microcrystalline cellulose (MCC), plant-based products and soil-based products (e.g., clays). Preferably, carriers are non-toxic to the recipient. In some instances the vaccines of the present invention, with or without carriers, can be presented to a recipient for ingestion via suspension in

drinking water. One of skill in the art is readily able to choose such carriers for application to recipient animals such as porcine.

**[0062]** Vaccines in the present invention may be modified-live vaccines (MLV), which are also referred to as attenuated vaccines. An MLV is a vaccine based on a virus modified such that it no longer causes the same clinical symptoms when administered to a recipient. For example, swine administered with a MLV for PRRSV would not exhibit the same clinical symptoms they would if they had been directly infected by PRRSV.

**[0063]** Vaccines in the present invention involve the editing of nucleotide and/or amino acid sequences. Though examples of only specific modifications, i.e. deletions or additions, of certain sequence lengths and in certain locations within the sequences are given below, one of ordinary skill in the art would understand that minor modifications may also be useful in the present invention. For example, a deletion may start up to 6 nucleotide residues prior to or after the disclosed starting location, and a deletion may end up to 6 nucleotide residues prior to or after the disclosed ending location. Further, an insertion, such as a tag, may be exactly the same length as one of the disclosed tags or  $\pm 4$  amino acids in length.

**[0064]** "Porcine" and "swine" are used interchangeably herein and refer to any animal that is a member of the family Suidae such as, for example, a pig.

**[0065]** Although the following Example uses a single parental strain, it is envisioned that the nsp2 region used in the present invention may be used as an insert and/or replacement in any other suitable viral strain.

### Examples

**[0066]** To produce vaccine viruses capable of differentiating infected from vaccinated animals (DIVA) and to be useful as tags to follow virus infection of cultured cells or in animals, two deletion constructs of the parental pMLV strain (as produced from the PRRSV strain VR-2332, as described in Wang, et al, 2008; “Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence,” *Virology* 371, 418-429) that had proven replication competent in MARC-145 cells [pV7-nsp2 $\Delta$ 23 (deletion of nsp2 N-terminal amino acids (aa) 12-34) and pV7-nsp2 $\Delta$ 87 (deletion of nsp2 hypervariable region aa 726-812)] were selected for further study. pV7-nsp2 $\Delta$ 87 has also been shown to be replication competent in swine, producing adequate titers (Faaberg et al., 2010. In vivo growth of porcine reproductive and respiratory syndrome virus engineered nsp2 deletion mutants. *Virus Res.* 154, 77-85; Guo et al., 2011. Large scale parallel pyrosequencing technology: PRRSV strain VR-2332 nsp2 deletion mutant stability in swine. *Virus Res.* 161, 162-169).

**[0067]** The cDNA sequence of the nsp2 region of the pMLV strain is given in SEQ ID NO: 1 and the cDNA sequences of nsp2 region of the two deletion constructs of nsp2 $\Delta$ 23 and nsp2 $\Delta$ 87 are given in SEQ ID NO: 2 and SEQ ID NO: 4, respectively. The amino acid sequences of the two deletion constructs of nsp2 $\Delta$ 23 and nsp2 $\Delta$ 87 are given in SEQ ID NO: 3 and SEQ ID NO: 5, respectively. Three small immunogenic tags were chosen for insertion into the deletion mutants: a small epitope found on the P and V proteins of simian virus 5 (V5), the commonly used synthetic FLAG tag, and an oligopeptide derived from pancreatic ribonuclease A (S-tag) (Fig. 1A).

**[0068]** To generate a simplified cloning vector that lacked critical restriction enzyme recognition sites, an existing cloning vector (pCR<sup>®</sup>-XL-TOPO; ThermoFisher Scientific,

Waltham, MA, USA) was deconstructed and modified. To do so, pCR<sup>®</sup>-XL-TOPO was digested with HinfI and the two fragments (339 bp, 1043 bp) containing the origin of replication and the Zeocin resistance gene were gel purified and ligated. Subsequently, a multiple cloning site (MCS) was inserted by ligation of four annealed synthetic oligonucleotides to encode the sequence

5-

CATGTACTAGTAGGCCTAGATCTGCATGCGAATTCCGCGGATCCGGCCGGCCGGCCG  
 GCCCGGGCTCTAGACTCGAGAAGCTTTTAATTAAACCATG (SEQ ID NO: 24) into the  
 existing PciI site. This vector was designated “pCHAZ.”

**[0069]** To generate subclones of the targeted regions of nsp2, 2-step mutagenic PCR was carried out using Phusion polymerase (New England Biolabs, Ipswich, MA, USA) and the pMLV infectious clone as a template. This mutagenic PCR introduced a pair of inverted EarI sites just internal to the region targeted for deletion, such that the sites excised themselves upon digestion and subsequent fill-in of the overhangs and self-ligation would generate the desired deletion. PCR products were A-tailed using GoTaq polymerase (Promega, Madison, WI, USA) and subcloned into pGEMTeasy (Promega). All PCR subclones were verified by sequencing. These PCR subclones were subsequently transferred into pCHAZ through ligation of the SphI/SpeI fragment from pGEMTeasy into the corresponding sites of the pCHAZ MCS. The  $\Delta 23$ /EarI and  $\Delta 87$ /EarI pCHAZ subclones were then digested with EarI and filled-in using Klenow DNA polymerase (New England Biolabs). To generate the untagged deletions, cut plasmids were self-ligated. To insert an epitope tag, complimentary 5'-phosphorylated synthetic oligonucleotides encoding the desired epitope tag were annealed and ligated in place of the deleted sequence. The oligonucleotide sequences encoding the tags for V5, S-Tag, and FLAG are given in SEQ ID NO: 6, 8, and 10, respectively. The amino acid sequences of each tag V5, S-Tag, and FLAG are given

in SEQ ID NO: 7, 9, and 11, respectively. Each oligonucleotide tag sequence was engineered to contain an identifying restriction enzyme recognition site (V5: XbaI; S-tag: NotI; 3xFLAG: PsiI), through translationally silent nucleotide changes. Once sequence verified, pCHAZ subclones were transferred to an intermediate clone containing nucleotides 1-4546 of the MLV genome, terminating in an FseI site, termed “MLV-Fragment I.” This transfer was completed through ligation of the EcoRI/BamHI ( $\Delta 23$ ) or HindIII/FseI ( $\Delta 87$ ) fragments from the respective deleted or tagged-deleted pCHAZ clones into the MLV-Fragment I intermediate clone. All intermediate clones were then transferred to the full-length pMLV infectious clone, by swapping the parental SphI/FseI fragment with the corresponding respective deleted or tagged-deleted sequence. Traditional Sanger sequencing using existing PRRSV primers (available on request) was used to determine the full-length sequence of all prepared pMLV mutant cDNA clones.

**[0070]** Two of the resulting viruses were negative DIVAs (rMLV-nsp2 $\Delta 23$  and rMLV-nsp2 $\Delta 87$ ) and six viruses were positive DIVAs (rMLV-nsp2 $\Delta 23$ -V5, rMLV-nsp2 $\Delta 23$ -S, rMLV-nsp2 $\Delta 23$ -FLAG, rMLV-nsp2 $\Delta 87$ -V5, rMLV-nsp2 $\Delta 87$ -S, rMLV-nsp2 $\Delta 87$ -FLAG). These virus names have been abbreviated herein to  $\Delta 23$ ,  $\Delta 87$ ,  $\Delta 23$ -V5,  $\Delta 23$ -S,  $\Delta 23$ -FLAG,  $\Delta 87$ -V5,  $\Delta 87$ -S,  $\Delta 87$ -FLAG, respectively.

**[0071]** The full-length cDNA clones (10  $\mu$ g) were linearized at the 3'-end by cleavage with PacI, extracted with phenol:chloroform and resuspended in 10  $\mu$ l RNase-free TE (10 mM Tris-HCl, 1 mM EDTA) or water. Capped RNA transcripts were produced from 1  $\mu$ g linearized DNA using the mMESSAGE MACHINE T7 Ultra kit (ThermoFisher Scientific). Transcribed RNA was then purified by phenol:chloroform extraction and resuspended in RNase-free water (40  $\mu$ l). RNA was evaluated for quality on 1% native agarose gel and quantified by spectrophotometry. Transcripts (10 ng or 100 ng) derived from pMLV $\Delta 23$ , pMLV $\Delta 87$ , pMLV $\Delta 23$ -V5, pMLV $\Delta 23$ -

FLAG and pMLVΔ87-V5 were initially transfected in MARC-145 cells using DMRIE-C (Han, et al., 2007. Identification of nonessential regions of the nsp2 replicase protein of porcine reproductive and respiratory syndrome virus strain VR-2332 for replication in cell culture. J. Virol. 81, 9878-9890).

**[0072]** MARC-145 cultured cells in Minimum Essential Medium Eagle (EMEM) medium (Sigma-Aldrich 56416c; St. Louis, MO, USA) supplemented with 10% fetal bovine serum (bovine viral diarrhea free; Omega Scientific Inc., Tarzana, CA, USA) and 50 µg/ml gentamicin sulfate was used to rescue and propagate recombinant viruses.

**[0073]** Subsequently, all RNA transcripts (1.5 and 3 µg) were electroporated in duplicate into MARC-145 cells using an established method (Ansari, et al., 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J. Virol. 80, 3994-4004). Electroporated MARC-145 cells were plated onto 60 cm<sup>2</sup> plates (passage 0) and passaged up to 2 times on 6-well plates until cytopathic effect (CPE) was seen. Recombinant virus was blindly passaged for 10 passages by inoculating 100 µl infected cell clarified supernatant diluted in 900 µl basal medium onto a monolayer of MARC-145 cells at 80% confluency in a T75 flask, rotating the infected cells 1 h at room temperature, then incubating the cells at 37°C, 5% CO<sub>2</sub> for 4-5 days. Each passage was harvested, centrifuged at 4000 rpm for 10 min, aliquoted into 2-4 ml tubes and frozen at -80°C.

**[0074] Confirmation of viral mutants.** In order to provide evidence that the modified viruses were as expected, RNA was purified from each virus at passage 3 and passage 10 and fully analyzed by RT-PCR (Figs. 1A and 1B). Viral RNA from infected cell supernatants at 3 and 10 passages (50 µl each of 9 viruses) was extracted using the MagMAX™ Total Nucleic Acid

Isolation Kit with a MagMAX™ Express Particle Processor (ThermoFisher Scientific) and subjected to analysis using the One-Step RT-PCR Kit (Qiagen, Valencia, CA). The thermocycler settings were as follows: 1. 50°C, 30 min for 1 cycle; 2. 95°C, 15 min for 1 cycle; 3. Thirty-five cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min. 4. 72°C, 10 min for 1 cycle; 5. Let sit at 4°C until further analysis.

**[0075]** After passage 3, primer pairs were utilized to confirm the expected size of each product (Fig. 1B, pairs A and C) and to verify that each virus was not contaminated by other mutant viruses or parental MLV (Fig. 1B, pairs B and D). The following primer pairs were utilized with 10µl viral RNA: A. 5'-GCGGAGGCTGCAAGTTAATGGTCTC (SEQ ID NO: 25)/5'-CGCAGGGAGTCTGAGGATTTGGATG (SEQ ID NO: 26); B. 5'-GCGGAGGCTGCAAGTTAATGGTCTC (SEQ ID NO: 27)/5'-CTTGGCCTGCCGGGTTTCACGA (SEQ ID NO: 28); C. 5'-GGGCATCTCCAAGAGGTAAAGGAAAC (SEQ ID NO: 29)/5'-GAGGCACAATAGAGTAAAAGCTGCAAAAC (SEQ ID NO: 30); D. 5'-GGGCATCTCCAAGAGGTAAAGGAAAC (SEQ ID NO: 31)/5'-CTGTGCCTGCGGACGGAGCTGATG (SEQ ID NO: 32). Primer sets A and C for passage 3 (Fig. 1A) and 10 vRNAs were utilized to ensure the correct band size for positive identification of the Δ23 and Δ87 mutants. Primer sets B and D using passage 3 vRNAs were used to show the presence (at the correct size) or the absence of MLV parent viral sequence (deletion) (Fig. 1A).

**[0076]** At passage 10, in order to assess whether the mutant viruses still displayed the proper mutation with the correct inserted restriction enzyme site, restriction enzyme analysis was performed on all mutants using the products of primer sets A and C (Fig. 1C). The products were

digested with appropriate restriction enzymes to provide additional verification. No evidence of contamination with other viruses was observed.

**[0077] Viral mutant stability.** To assess the influence of viral passage on sequence stability as well as to reconfirm the passages were not contaminated with other mutants, viruses at passages 3 and 10 were also processed for next generation whole viral genome sequencing using the Illumina MiSeq desktop genome sequencer (Fig. 2). Geneious version 8.1.8 (<http://www.geneious.com>) was implemented to analyze the MiSeq reads in reference to the parent deletion or deletion-tagged virus, and to generate all nucleotide sequence alignments.

**[0078]** Overall, the modified viral genomes were stable and the mutations were few in number, except for  $\Delta 87$ -FLAG. All mutations but one led to amino acid changes, and all but one were single nucleotide polymorphisms (SNP). No changes were seen in the nucleotides that were modified by deletion or deletion-tagged insertion. Sixteen of the 35 mutations detected in the combined nucleotide alignment maintained the original nucleotide at a particular site, but also revealed a co-existing viral population with another nucleotide at that same site (Table 3). Only one nucleotide (ORF4 nt 13581) was shown to change consistently in most modified viruses, resulting in either a G→A, G→A/G (R) or evidence of two successive mutations A→A/T (W). Most amino acid mutations that arose over 3 to 10 passages of the modified viruses in MARC-145 cells reflected non-conservative changes. The mutation noted in ORF5 and ORF5a, seen in both  $\Delta 23$  and  $\Delta 87$ , removed a predicted N-glycosylation motif near the N-terminal end of GP5.

Table 3: Mutations arising at 3 and 10 passages in each DIVA vaccine

| Region       | Nucleotide | MLV | $\Delta 23$ |   |    | $\Delta 23$ -V5 |   |    | $\Delta 23$ -FLAG |   |    | $\Delta 23$ -S |   |    | $\Delta 87$ |   |    | $\Delta 87$ -V5 |   |    | $\Delta 87$ -FLAG |   |    | $\Delta 87$ -S |   |    | Amino acid mutation |
|--------------|------------|-----|-------------|---|----|-----------------|---|----|-------------------|---|----|----------------|---|----|-------------|---|----|-----------------|---|----|-------------------|---|----|----------------|---|----|---------------------|
|              |            |     | 0           | 3 | 10 | 0               | 3 | 10 | 0                 | 3 | 10 | 0              | 3 | 10 | 0           | 3 | 10 | 0               | 3 | 10 | 0                 | 3 | 10 | 0              | 3 | 10 |                     |
| Nsp1 $\beta$ | 953        | C   |             |   |    |                 |   |    |                   |   |    |                |   |    |             | Y |    |                 |   |    |                   |   |    |                |   |    | R→R/C               |
| Nsp1 $\beta$ | 1254       | T   |             |   |    |                 |   |    |                   |   | W  |                |   |    |             |   |    |                 |   |    |                   |   |    |                |   |    | L→L/H               |
| Nsp2         | 1342       | G   |             |   |    |                 |   |    |                   | A | A  |                |   |    |             |   |    |                 |   |    |                   |   |    |                |   |    | G→R                 |
| Nsp2         | 1883       | A   |             |   |    |                 |   |    |                   |   |    |                |   |    | R           |   |    |                 |   |    |                   |   |    |                |   |    | I→I/V               |

|             |       |   |   |   |   |   |   |   |   |   |   |        |
|-------------|-------|---|---|---|---|---|---|---|---|---|---|--------|
| Nsp2        | 2837  | A |   |   |   |   |   | R |   |   |   | S→S/G  |
| Nsp2        | 3291  | G |   |   |   |   |   |   | T |   |   | G→V    |
| Nsp2        | 3783  | C |   |   |   |   | Y |   |   |   |   | T→T/I  |
| Nsp4        | 5751  | C |   |   |   |   |   |   |   | T |   | S→L    |
| Nsp4        | 6146  | A |   | G |   | G |   |   |   |   |   | K→E    |
| Nsp7α       | 7200  | C |   |   |   | S |   |   |   |   |   | P→P/R  |
| Nsp7β       | 7201  | C |   |   |   | Y |   |   |   |   |   | S→S/G  |
| ORF2        | 12494 | A |   |   |   | G |   |   |   |   | S | Q→R    |
| ORF2        | 12639 | A |   |   |   | G |   |   |   |   |   | I→I/V  |
| ORF3        | 12976 | A |   | R |   | R |   |   |   |   |   | V→V//F |
| ORF4        | 13581 | G | A | A | A | A | A | A | W | W | W | T→T    |
| ORF4        | 13739 | T |   |   |   |   |   |   |   | Y | R | N→N/D; |
| ORF5;<br>5a | 13885 | A |   |   | R |   |   |   | R |   |   | Q→Q/R  |

**[0079] Northern blot analyses.** In PRRSV infected cells, many subgenomic RNA (sgRNA) species are detected that are produced by a discontinuous transcription strategy that proceeds through a fusion of the viral genome 5' untranslated region (UTR) to one of several sites downstream during negative strand synthesis (Kappes and Faaberg, 2015. PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. *Virology* 479-480, 475-486; Snijder et al., Arterivirus molecular biology and pathogenesis. *J. Gen. Virol.* 94, 2141-2163). These mRNAs include the standard sgRNAs (RNAs 2-7; Fig. 3 – ORF7) that encode the major and minor envelope proteins (glycoproteins GP2-5, unglycosylated envelope proteins E, ORF5a protein, matrix and nucleocapsid) that utilize an antisense transcription-regulating sequence (TRS) upstream of the 5' end of the respective protein coding region with a conserved TRS sequence (UUAACC) located at the end of the 5'UTR (Snijder et al., 2013).

**[0080]** Northern blot analysis was performed according to the NorthernMax® Kit instructions (AM1940; ThermoFisher Scientific). MARC-145 cells were inoculated with the third passage of each virus at an MOI of 0.01. Two days after infection, intracellular RNA was extracted, denatured for 1 hour at 50°C with glyoxal loading dye and then electrophoresed (5 µg/sample) on a glyoxal denaturing gel and transferred onto a 0.45 µm Whatman® Nytran™ SuPerCharge

nylon membrane (Sigma-Aldrich) by gravity overnight at room temperature. The membrane was hybridized with a digoxin-labeled oligonucleotide probe (7-p14890: 5'-CCTTCTTTCTCTTCTGCTGCTTGCCGTTGTTATTTGGCAT(SEQ ID NO: 33); V5: 5'-GGTGGAATCTAGACCCAGCAGTGGGTTGGGGATGGGCTTGCC(SEQ ID NO: 34); FLAG: 5'-CTTGTCATCGTCATCCTTGTAGTCGATATCGTGATCCTTGTAATCGCCGTCATGATCC TTATAATC(SEQ ID NO: 35); S: 5'-AATCCATATGCTGGCGTTCGAACTTAGCGGCC(SEQ ID NO: 36)) complementary to ORF7, V5, FLAG, and S-tag respectively in ULTRAhyb® Ultrasensitive Hybridization Buffer (AM8670; ThermoFisher Scientific) at 68°C for 16-20h, washed two times in low stringency 2X Saline-Sodium Citrate (0.3 M sodium chloride and 30 mM trisodium citrate; SSC) with 0.1% SDS at room temperature for 10 min. The membrane was then processed for immunological detection using the DIG DNA Labeling and Detection Kit following the manufacturer's instructions (11093657910 ROCHE; Sigma-Aldrich).

**[0081]** With some PRRSV strains, as is the case with MLV, other atypical RNAs are detected (Wang et al., 2008; Yuan et al., 2000; Yuan et al., 2004). These unusual RNAs, termed heteroclites, have been shown to be products of aberrant fusions of viral genome 5' sequence with downstream sequence at 2-7 nucleotide stretches that are not conserved and vary between the individual nonconforming subgenomic RNAs (Fig. 3, ORF). When the intracellular RNAs are probed with the Tag antisense oligomers, the tagged  $\Delta 23$  viral mutant RNAs (Fig. V5: lane 3; FLAG: lane 4; S-tag: lane 5) are detected in full-length transcripts as well as some of these heteroclite RNA species. However, only the full-length RNA is predominantly detected when the probes react with the tagged  $\Delta 87$  mutant intracellular RNA (Fig. 3, V5: lane 7; FLAG: lane 8; S-

tag; lane 9). The instability of  $\Delta 87$ -FLAG in this experiment resulted in the lack of hybridization to the negative FLAG oligonucleotide probe.

**[0082] Multi-step growth curve.** Using passage 3 stocks, the deletion and deletion-tagged viruses were analyzed for viral replication rate. Growth curves were performed simultaneously (0.1 MOI input) with sampling at 0, 6, 12, 24, 36, 48 and 72 h, using known techniques (Han et al., 2007. Identification of nonessential regions of the nsp2 replicase protein of porcine reproductive and respiratory syndrome virus strain VR-2332 for replication in cell culture. *J. Virol.* 81, 9878-9890; Wang et al., 2008). Virus titers of recombinant MLV,  $\Delta 23$ ,  $\Delta 87$ ,  $\Delta 23$ -V5,  $\Delta 87$ -V5,  $\Delta 23$ -S,  $\Delta 87$ -S,  $\Delta 23$ -FLAG and  $\Delta 87$ -FLAG at each time point were determined by plaque assay on MARC-145 cells. Titers (plaque forming units (PFU)/ml) were determined on MARC-145 cells to assess in vitro growth properties.

**[0083]** A range of replication rates was observed.  $\Delta 23$ ,  $\Delta 23$ -V5,  $\Delta 23$ -S,  $\Delta 23$ -FLAG and  $\Delta 87$ -V5 viruses replicated to titers that were 0.8-1.5 logs higher than MLV on MARC-145 cells, while  $\Delta 87$ ,  $\Delta 87$ -S,  $\Delta 87$ -FLAG viruses replicated at rates similar to the vaccine strain MLV (Fig. 4A). This was also evidenced by the plaque assay, which showed that all mutants gave similar or slightly larger plaques sizes than the parent MLV (Fig. 4B).

**[0084] Immunofluorescence.** Each tagged mutant was assessed by immunofluorescence with either an antibody to PRRSV nucleocapsid (SDOW17) or by an antibody to one of the three different tags. Uninfected or infected MARC-145 cells grown on chamber slides were fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS), and treated with 0.1% sodium borohydride PBS. Primary antibodies used were mAb SDOW-17 (1:50; RTI, Brookings, SD, USA) specific for the PRRSV nucleocapsid protein, mAb to the V5-tag (1:500; Abcam, Cambridge, MA, USA), mAb to the FLAG tag (1:500; Sigma-Aldrich, St.

Louis, MO) and mAb to the S tag (1:100; Abcam). The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated affinity isolated goat anti-mouse IgG (Fc specific; Sigma-Aldrich). DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclei (Thermo-Scientific). Stained slides were visualized using 40x and 60x oil immersion objectives on a Nikon A1R+ laser scanning confocal microscope.

**[0085]** All viral mutants showed reactivity to the nucleocapsid antibody, as shown with untagged deletion mutant  $\Delta 23$  (Fig. 4C). Each viral mutant detected with its respective antibody showed similar perinuclear fluorescence. In addition,  $\Delta 23$ -V5,  $\Delta 23$ -S, and  $\Delta 23$ -FLAG were shown to distribute into a diffuse punctate pattern, characteristic of endoplasmic reticulum (ER)/Golgi compartments, similar to results generated previously with an  $\alpha$ -nsp2-OTU antibody (Kappes et al., 2013. Highly divergent strains of porcine reproductive and respiratory syndrome virus incorporate multiple isoforms of nonstructural protein 2 into virions. J. Virol. 87, 13456-13465). Since the small immunogenic tags were placed within the recognized OTU domain (nsp2 aa 1-215), these results are both consistent and suggest that modification of the N-terminal region does not interfere with viral protein processing or cellular localization. Detection of  $\Delta 87$ -V5,  $\Delta 87$ -S, and  $\Delta 87$ -FLAG by their respective antibodies showed that the hypervariable region of nsp2 was more localized to regions directly adjacent to the infected cell nuclei, although a minor amount of nsp2 was also detected in the ER/Golgi region (Fig. 4C). No cross reactivity between the deletion and deletion-tagged viruses was detected when stained with a tag antibody different from the antibody to the engineered tag of each genetically altered virus, showing that these separate viruses could potentially be co-infected without loss of specificity (data not shown). Co-infection of MARC-145 cells with  $\Delta 23$ -S and  $\Delta 87$ -V5 tagged DIVAs revealed some differentiation in non-perinuclear localization via an immunofluorescence assay (Fig. 4D).

[0086] The foregoing description and accompanying figures illustrate the principles, preferred embodiments and modes of operation of the invention. However, the invention should not be construed as being limited to the particular embodiments discussed above. Additional variations of the embodiments discussed above will be appreciated by those skilled in the art.

[0087] Therefore, the above-described embodiments should be regarded as illustrative rather than restrictive. Accordingly, it should be appreciated that variations to those embodiments can be made by those skilled in the art without departing from the scope of the invention as defined by the following claims.

**CLAIMS**

What is claimed is:

Claim 1: An immunogenic composition, comprising an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region,

wherein the nsp2 region has a modification at one of amino acids 10-14,

wherein the modification comprises a deletion, and

wherein the immunogenic composition is capable of inducing an immune response in a recipient.

Claim 2: The immunogenic composition of claim 1, further comprising a pharmaceutically acceptable adjuvant.

Claim 3: The immunogenic composition of claim 1, further comprising a pharmaceutically acceptable carrier.

Claim 4: The immunogenic composition of claim 1, wherein the modification is at amino acid 12.

Claim 5: The immunogenic composition of claim 1, wherein the amino acid sequence of the nsp2 region is one of a sequence of SEQ ID NO: 3 and a sequence having at least 95% homology to SEQ ID NO: 3.

Claim 6: The immunogenic composition of claim 1, wherein the modification further comprises an insertion, and

wherein the insertion comprises the insertion of an epitope tag of 10-25 amino acids in length.

Claim 7: The immunogenic composition of claim 6, wherein the epitope tag is one of a V5 tag, an S-Tag, and a FLAG tag.

Claim 8: The immunogenic composition of claim 7, wherein the amino acid sequence of the nsp2 region is one of a sequence of SEQ ID NO: 13, a sequence of SEQ ID NO: 15, a sequence of SEQ ID NO: 17, a sequence having at least 95% homology to SEQ ID NO: 13, a sequence having at least 95% homology to SEQ ID NO: 15, and a sequence having at least 95% homology to SEQ ID NO: 17.

Claim 9: An immunogenic composition, comprising an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region,

wherein the nsp2 region has a modification at amino acid 724-728,

wherein the modification comprises a deletion, and

wherein the immunogenic composition is capable of inducing an immune response in a recipient.

Claim 10: The immunogenic composition of claim 9, wherein the modification is at amino acid 726.

Claim 11: The immunogenic composition of claim 9, wherein the amino acid sequence of the nsp2 region is one of a sequence of SEQ ID NO: 5 and a sequence having at least 95% homology to SEQ ID NO: 5.

Claim 12: The immunogenic composition of claim 9, wherein the modification further comprises an insertion, and

wherein the insertion comprises the insertion of an epitope tag of 10-25 amino acids in length.

Claim 13: The immunogenic composition of claim 12, wherein the epitope tag is one of a V5 tag, an S-Tag, and a FLAG tag.

Claim 14: The immunogenic composition of claim 13, wherein the amino acid sequence of the nsp2 region is one of a sequence of SEQ ID NO: 19, a sequence of SEQ ID NO: 21, a sequence of SEQ ID NO: 23, a sequence having at least 95% homology to SEQ ID NO: 19, a sequence having at least 95% homology to SEQ ID NO: 21, and a sequence having at least 95% homology to SEQ ID NO: 23.

Claim 15: The immunogenic composition of claim 9, further comprising a pharmaceutically acceptable adjuvant.

Claim 16: The immunogenic composition of claim 9, further comprising a pharmaceutically acceptable carrier.

Claim 17: An immunogenic composition, comprising an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region,

wherein the nsp2 region comprises a deletion at a deletion starting point, the deletion starting point being one of amino acids 10-14 or one of amino acids 724-728, the deletion comprising the removal of 21-89 amino acids, and

wherein the nsp2 region optionally comprises an insertion of an epitope tag of 10-25 amino acids in length at the deletion starting point.

Claim 18: A method for reducing the incidence of porcine reproductive and respiratory syndrome virus (PRRSV) in swine, comprising administering to a swine an immunogenic composition in an amount effective to generate an immune response in said swine to said PRRSV,

wherein the immunogenic composition comprises an isolated polynucleotide sequence representing a modified live vaccine for porcine reproductive and respiratory syndrome virus having an nsp2 region,

wherein the nsp2 region has a modification at one of amino acids 10-14 or one of amino acids 724-728, and

wherein the modification comprises a deletion.

Claim 19: The method for reducing the incidence of PRRSV in swine of claim 18, wherein the immune response is protection against porcine reproductive and respiratory syndrome virus.

Claim 20: The method for reducing the incidence of PRRSV in swine of claim 18, wherein the modification further comprises an insertion, and

wherein the insertion comprises the insertion of an epitope tag of 10-25 amino acids in length.

Claim 21: The method for reducing the incidence of PRRSV in swine of claim 20, wherein the immune response is protection against porcine reproductive and respiratory syndrome virus.

\* \* \* \*

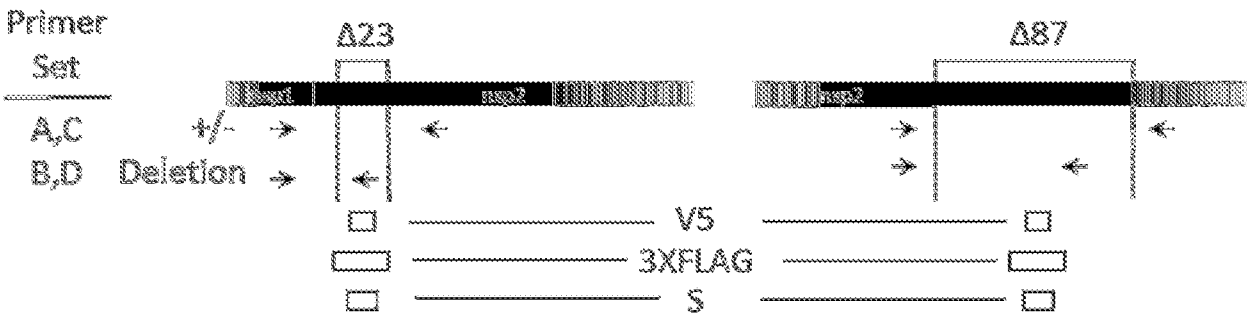


FIG. 1A

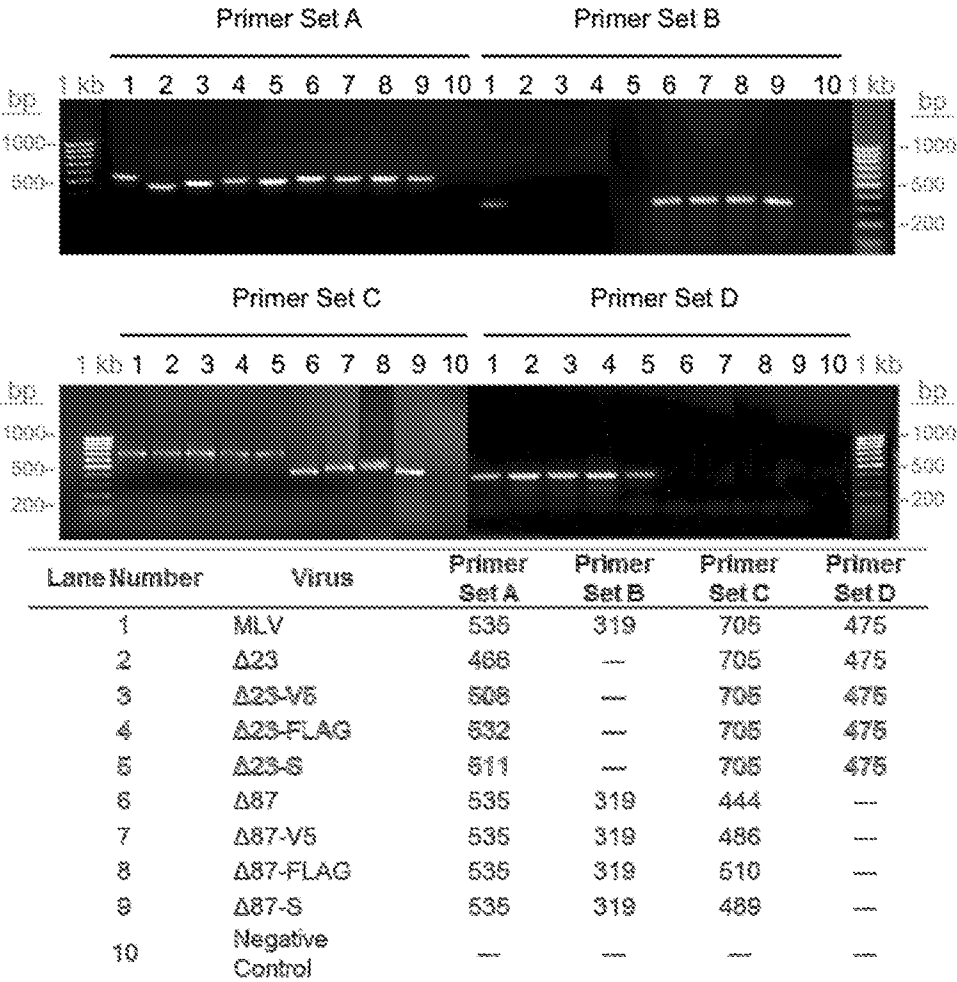
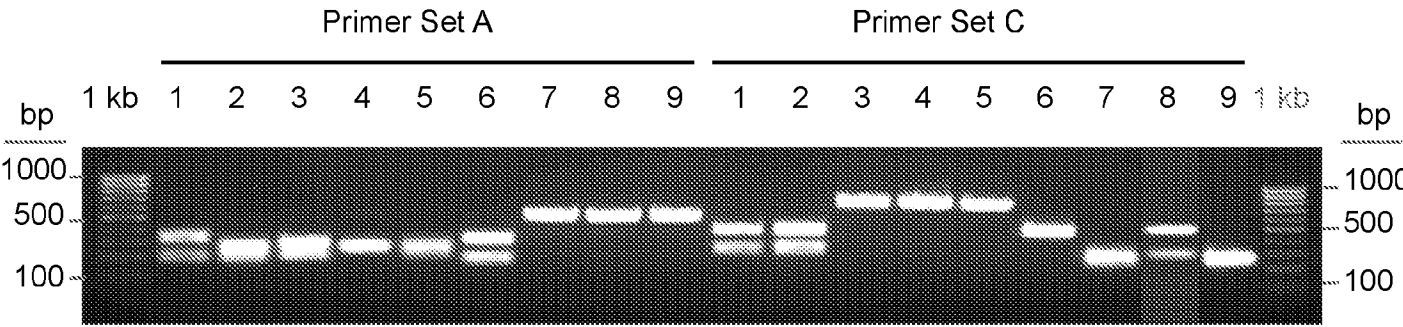


FIG. 1B



| Lane Number | Virus    | Primer Set A | Enzyme | Fragment Sizes | Primer Set C | Enzyme | Fragment Sizes |
|-------------|----------|--------------|--------|----------------|--------------|--------|----------------|
| 1           | MLV      | 535          | NaeI   | 203, 332       | 705          | EcoRV  | 280, 426       |
| 2           | Δ23      | 488          | NaeI   | 203, 263       | 705          | EcoRV  | 280, 426       |
| 3           | Δ23-V5   | 508          | XbaI   | 218, 292       | 705          | XbaI   | 705            |
| 4           | Δ23-FLAG | 532          | PstI   | 265, 270       | 705          | PstI   | 705            |
| 5           | Δ23-S    | 511          | NotI   | 238, 273       | 705          | NotI   | 705            |
| 6           | Δ87      | 535          | NaeI   | 203, 332       | 444          | EcoRV  | 444            |
| 7           | Δ87-V5   | 535          | XbaI   | 535            | 488          | XbaI   | 231, 258       |
| 8           | Δ87-FLAG | 535          | PstI   | 535            | 510          | PstI   | 230, 280       |
| 9           | Δ87-S    | 535          | NotI   | 535            | 488          | NotI   | 236, 283       |

FIG. 1C

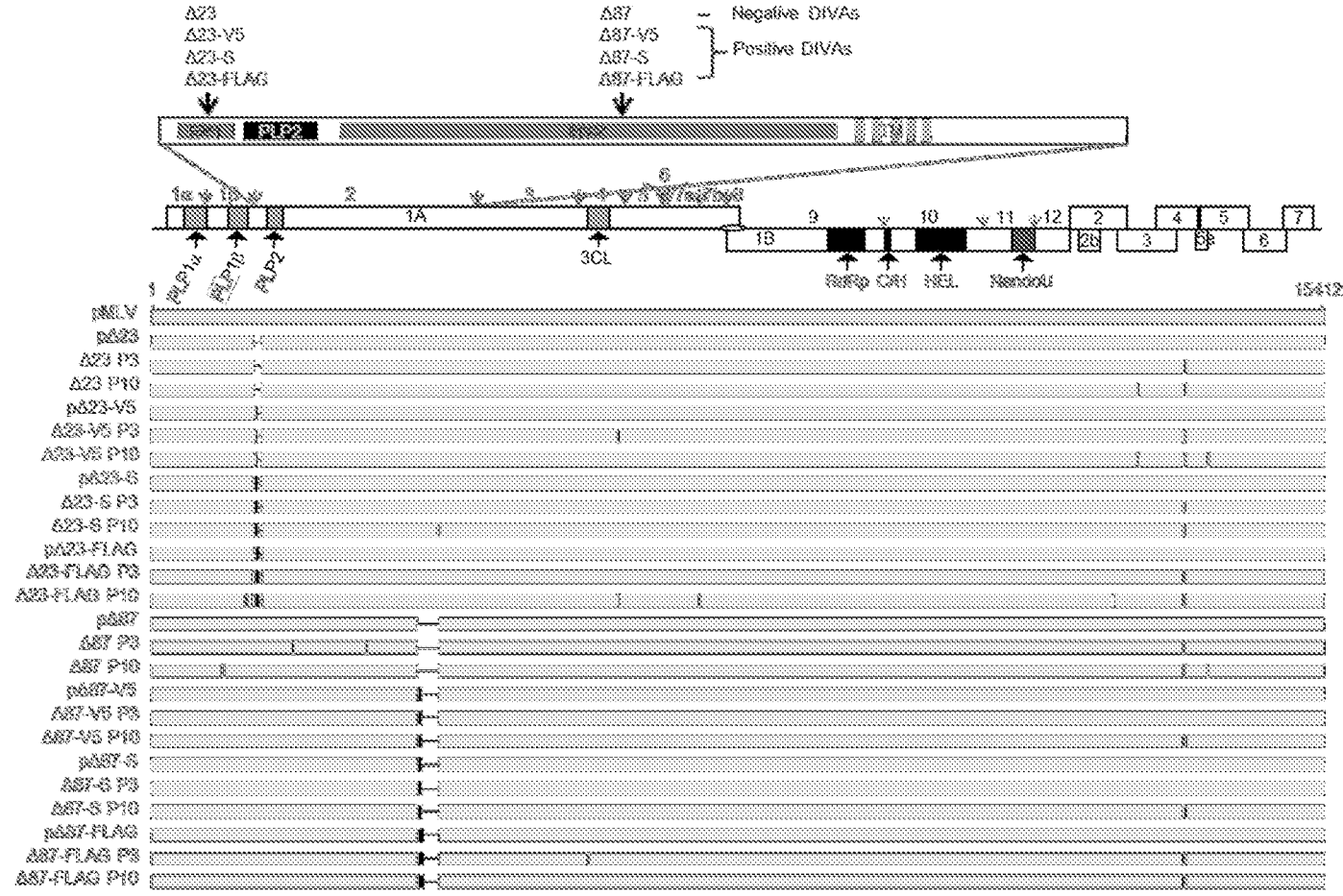


FIG. 2

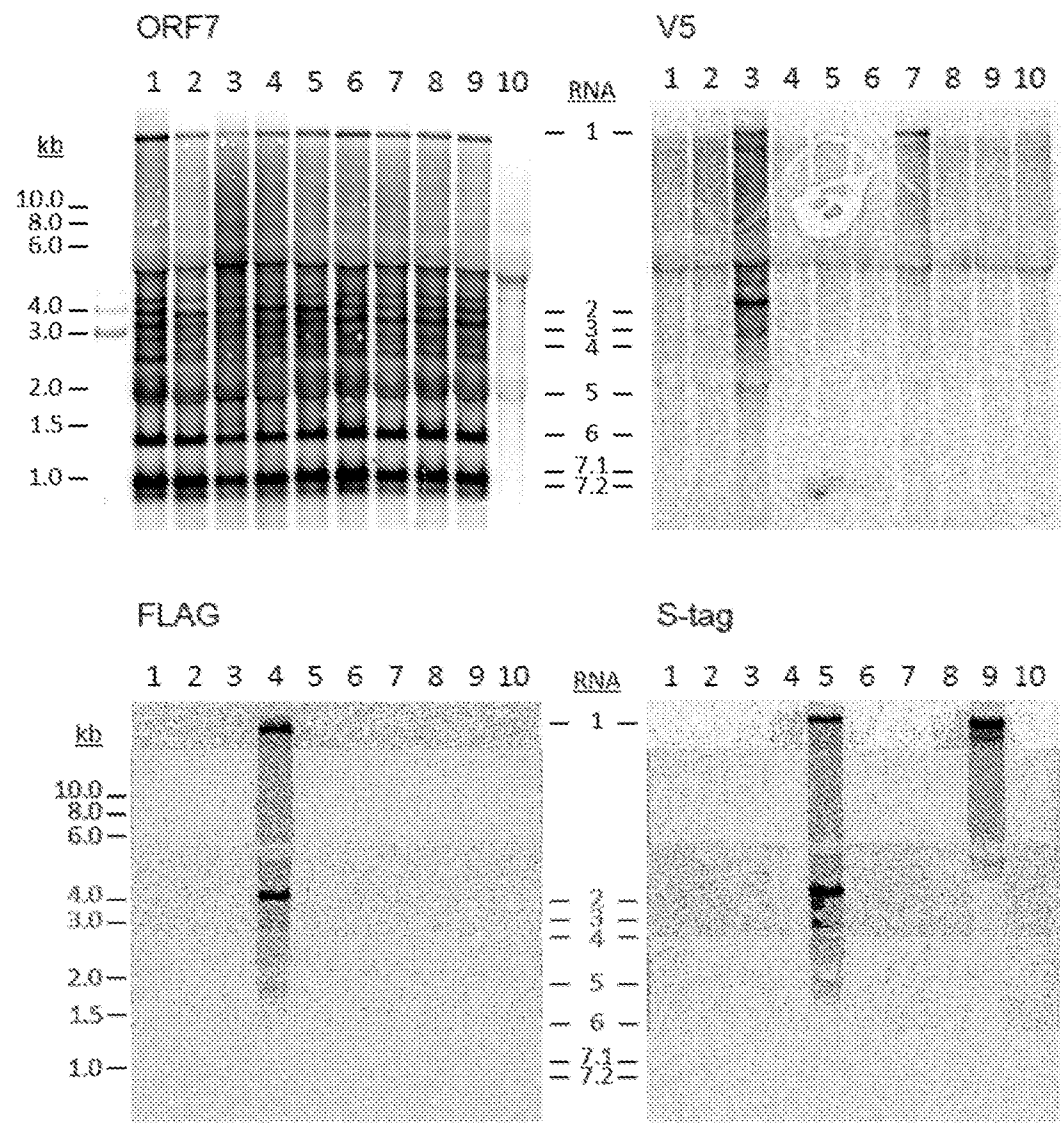


FIG. 3

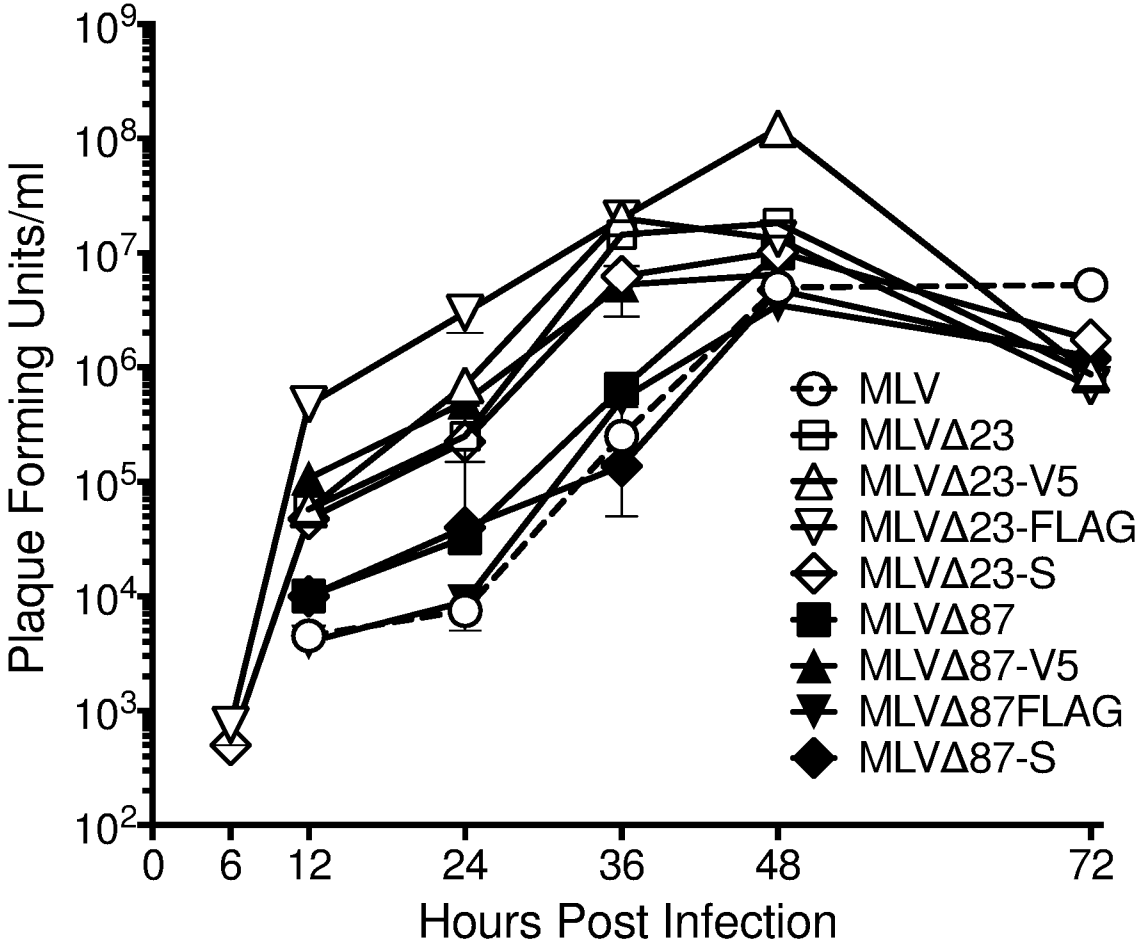


FIG. 4A

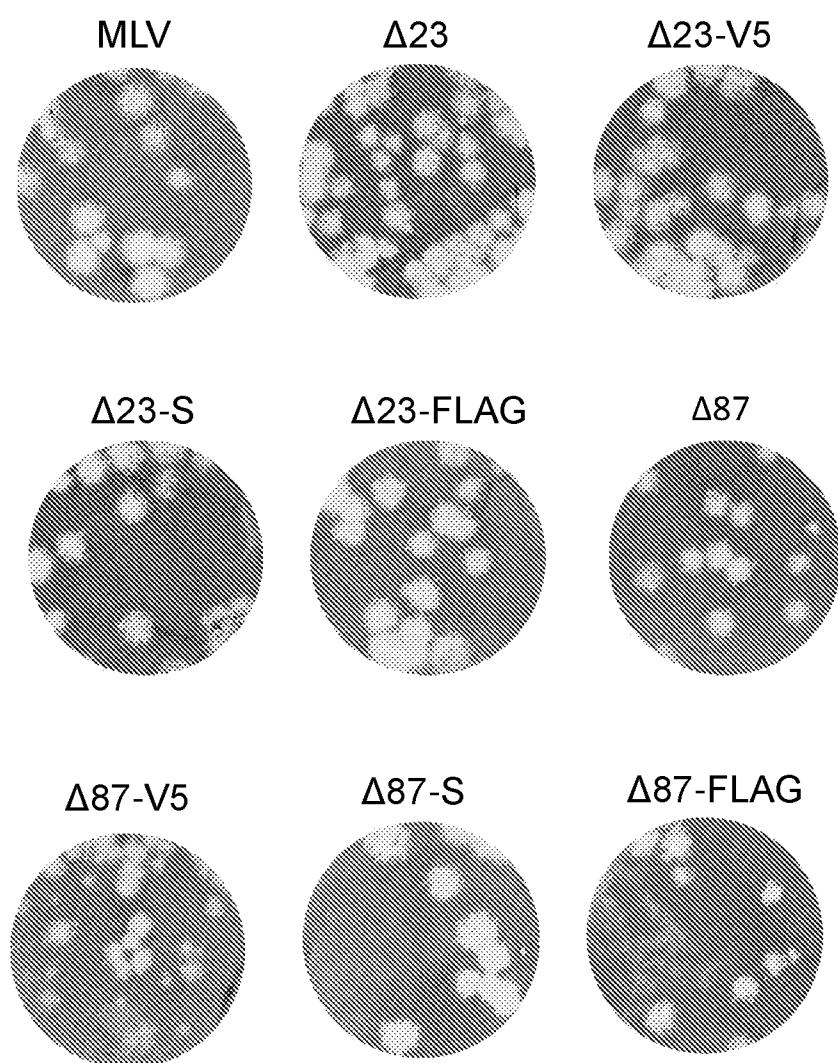


FIG. 4B

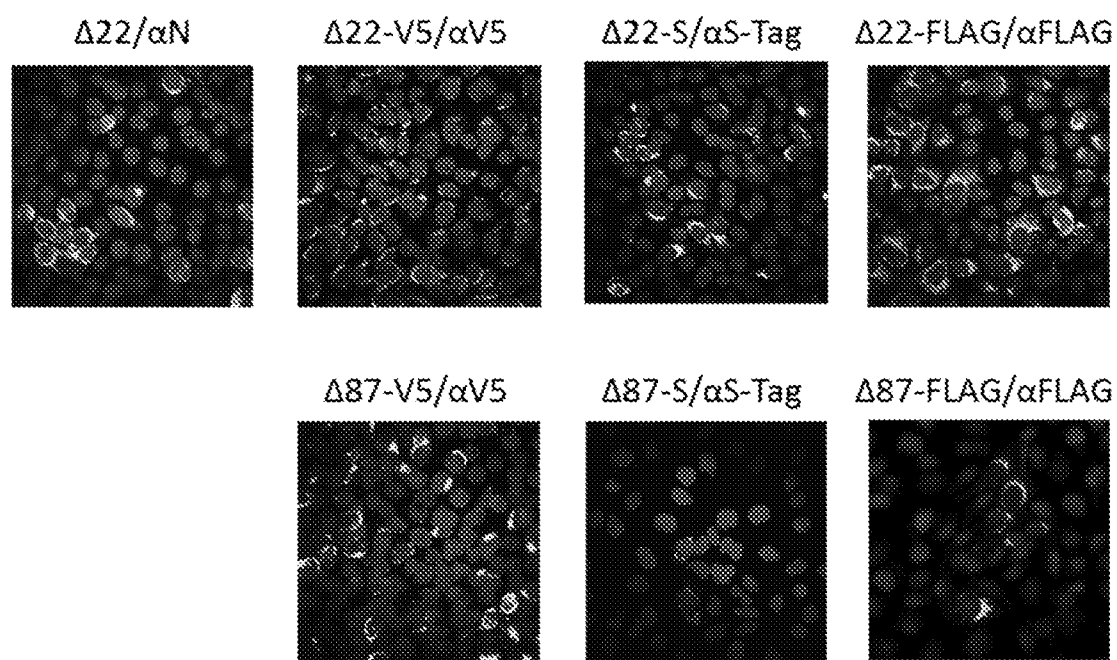


FIG. 4C

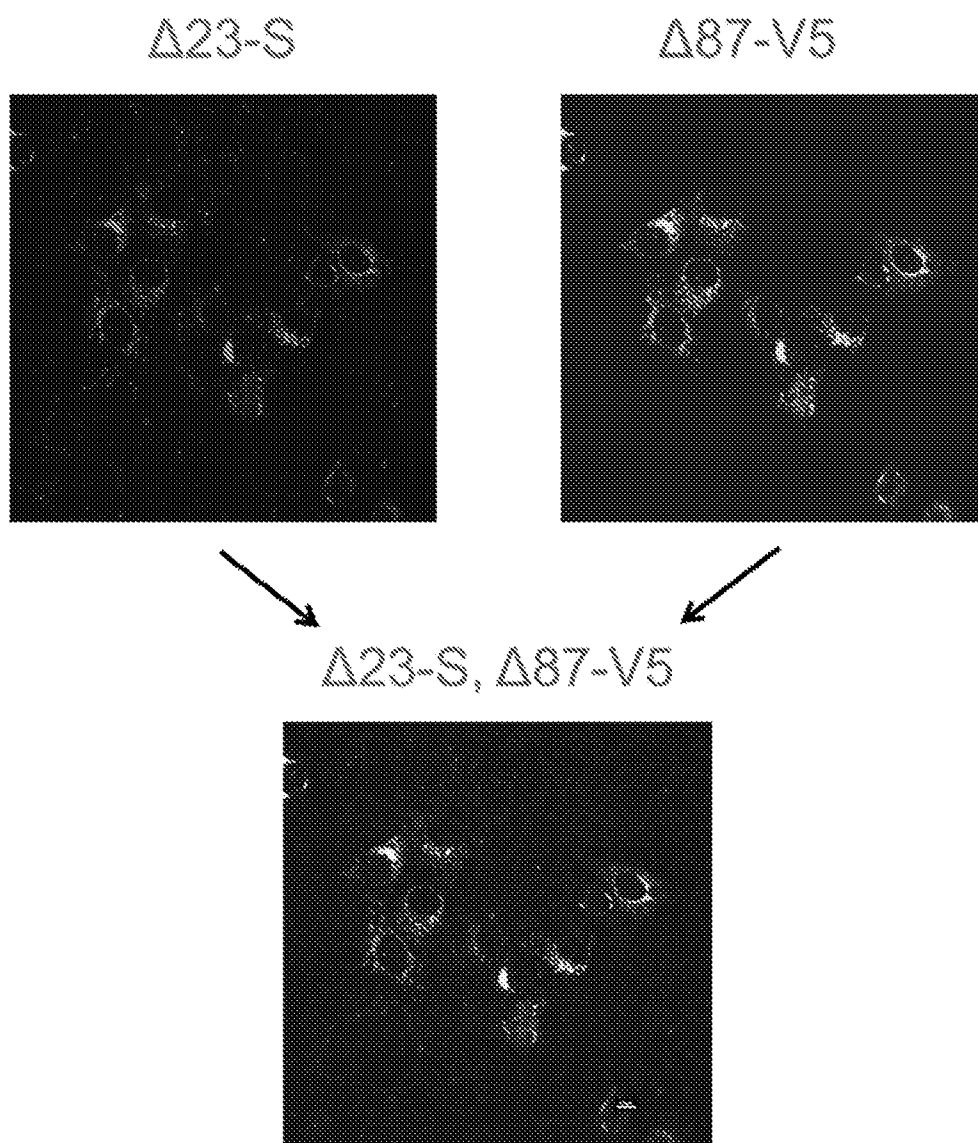


FIG. 4D

**A. CLASSIFICATION OF SUBJECT MATTER****A61K 39/12(2006.01)i, A61K 39/39(2006.01)i, A61P 31/12(2006.01)i, A61K 39/00(2006.01)i**

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

**B. FIELDS SEARCHED**Minimum documentation searched (classification system followed by classification symbols)  
A61K 39/12; A61P 31/14; C07H 21/04; C07K 2/00; A61K 39/39; A61P 31/12; A61K 39/00Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Korean utility models and applications for utility models  
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
eKOMPASS(KIPO internal) & Keywords: Porcine reproductive and respiratory syndrome virus, vaccine, nsp2 region, deletion, epitope tag**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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



See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

15 October 2018 (15.10.2018)

Date of mailing of the international search report

**15 October 2018 (15.10.2018)**

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

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# INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/US2018/034917**

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

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

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

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

3. Additional comments:

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2018/034917**

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Information on patent family members

International application No.

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