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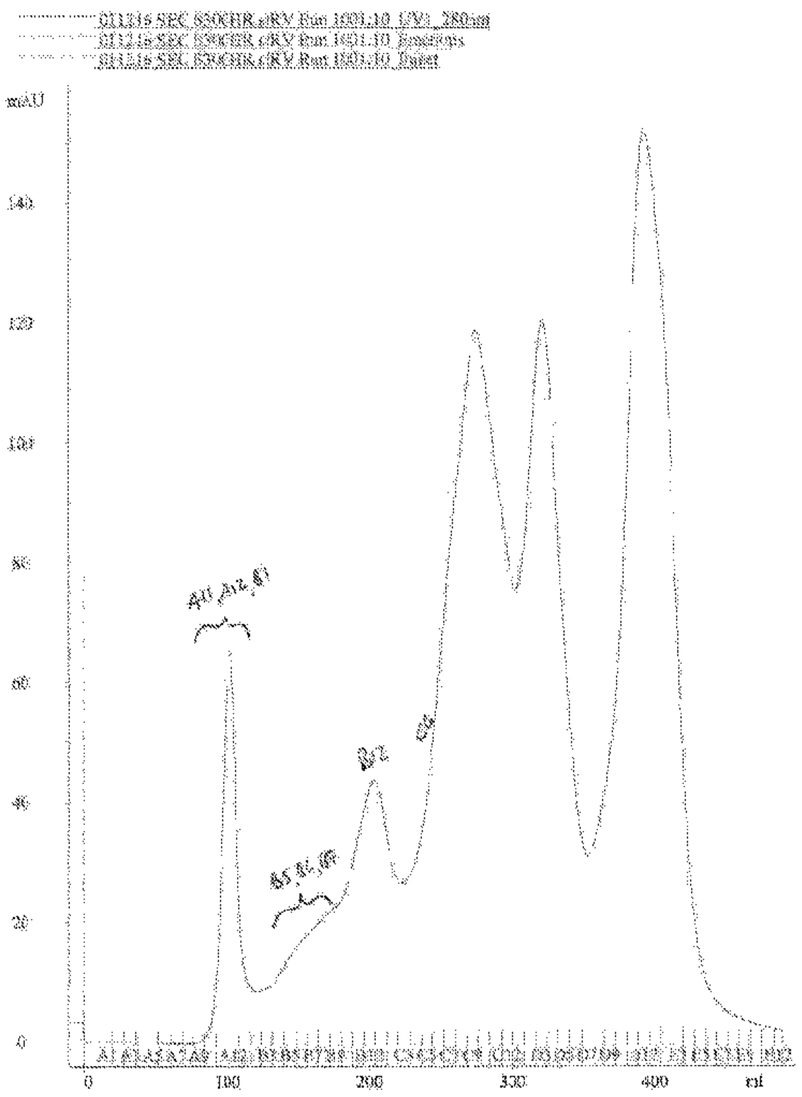
(54) Title: MARKER SYSTEM, IN PARTICULAR FOR BACULOVIRUS-EXPRESSED SUBUNIT ANTIGENS

FIG. 6



(57) Abrégé/Abstract:
The present invention belongs to the field of compliance markers and marker vaccines which allow for the differentiation between infected and vaccinated individuals. In particular, it relates to a method of determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells.

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[Continued on next page]

(54) **Title**: MARKER SYSTEM, IN PARTICULAR FOR BACULOVIRUS-EXPRESSED SUBUNIT ANTIGENS

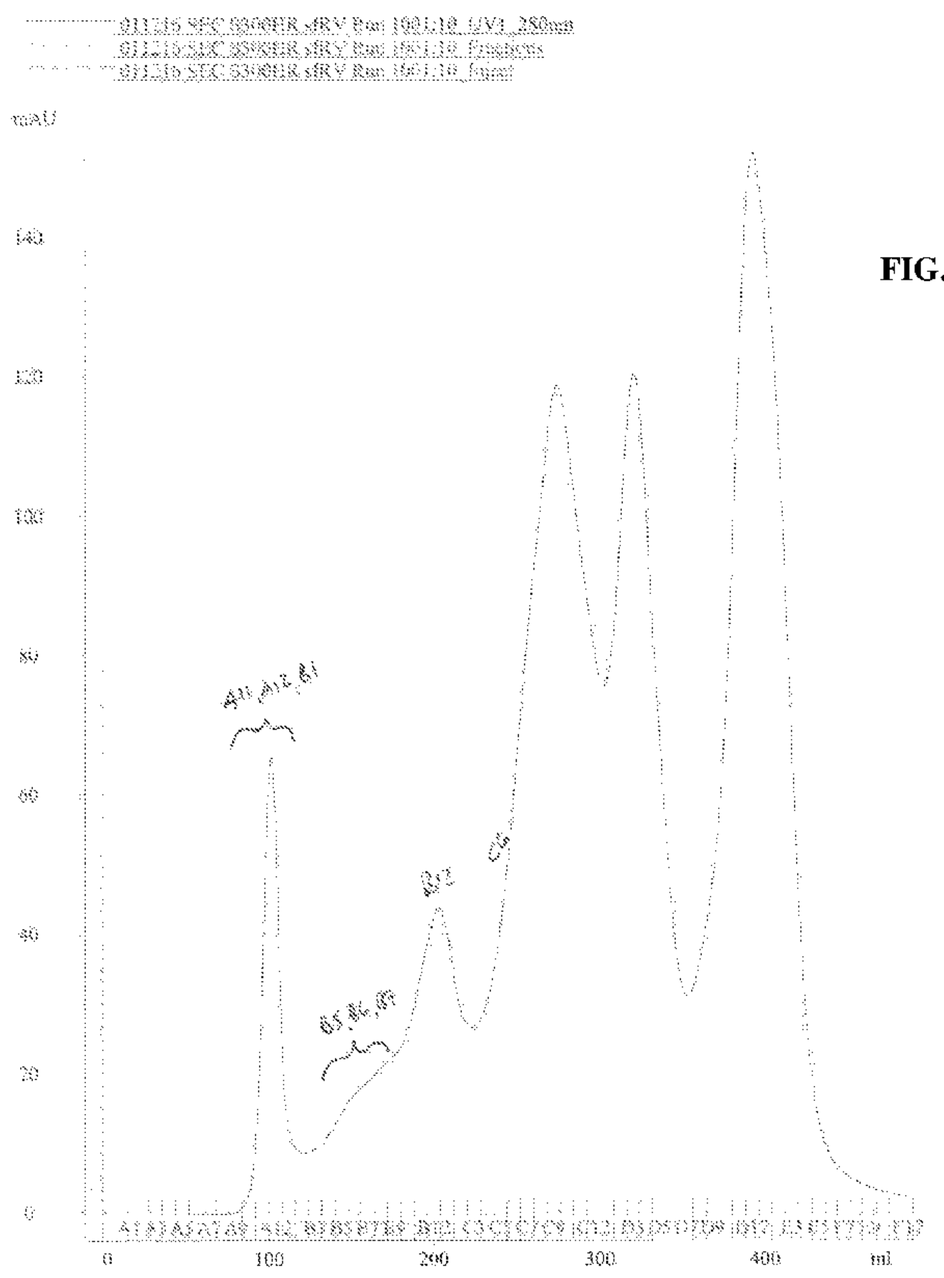

FIG. 6

(57) **Abstract**: The present invention belongs to the field of compliance markers and marker vaccines which allow for the differentiation between infected and vaccinated individuals. In particular, it relates to a method of determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells.

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MARKER SYSTEM, IN PARTICULAR FOR BACULOVIRUS-EXPRESSED SUBUNIT ANTIGENS

SEQUENCE LISTING

[0001] This application contains a sequence listing in accordance with 37 C.F.R. 1.821 – 1.825. The sequence listing accompanying this application is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention belongs to the field of compliance markers and marker vaccines which allow for the differentiation between infected and vaccinated individuals. In particular, it relates to a compliance marker for vaccines including a subunit antigen, and a DIVA (Differentiating Infected from Vaccinated Animals) system which makes it possible to differentiate between animals infected with a pathogen and animals treated with a subunit antigen derived from said pathogen, wherein said subunit antigen has been expressed in cultured insect cells, preferably by means of a genetically engineered baculovirus.

Description of the Related Art

[0003] Baculoviruses are large rod-shaped double stranded DNA viruses that infect invertebrates, in particular insects, but do not replicate in mammalian or other vertebrate cells. Starting in the 1940´s they were first used as biopesticides in the crop fields. Additionally, after the publication of a first paper describing the overexpression of human beta interferon in insect cells (Smith et al. Mol Cell Biol. 3: 2156–2165 (1983)), genetically engineered baculoviruses have been widely used for producing complex recombinant proteins in insect cell cultures, including the production of antigens for several approved human and veterinary subunit vaccines (van Oers et al. J Gen Virol. 96: 6-23 (2015)).

[0004] Vaccination is an essential tool to manage herd health, in particular in high density confinement settings where many food animals are raised. When disease outbreaks occur in animals that were supposedly vaccinated, questions arise as to whether the vaccine failed to protect the animals or whether the vaccine was delivered properly, wherein the latter possibility regarding proper delivery of the vaccine is referred to as vaccine compliance.

[0005] The use of compliance markers for determining if an animal has been properly vaccinated is thus highly desired by producers. WO 2009/058835 A1 describes e.g. the use of purified xylanase which was added as a compliance marker to a swine influenza vaccine. Regarding vaccines comprising baculovirus-expressed subunit antigens, it is possible to use baculovirus antigens as a compliance marker, wherein, however, currently used systems have limitations in animals testing positive and that a high amount of antigen is needed (Gerber *et al.* Res Vet Sci. 95:775-81 (2013); Lehnert. Top Agrar 5: S11-S14 (2011)).

[0006] Vaccines used in programs for controlling viral outbreaks and infections must have an effective system to monitor for continued presence of viral infection within the population. However, vaccination complicates large scale surveillance for the spread of the infection by e.g. serological means, as both vaccinated and exposed individuals produce antibody specific for the virus. The antigenic similarity between the infecting virulent field strain of the virus and the viral vaccine frequently hampers the discrimination between infected and vaccinated subjects as vaccination results in the occurrence and persistence of antibodies that are indistinguishable between infected and vaccinated individuals.

[0007] There is increasing worldwide interest in DIVA (differentiating infected and vaccinated animals) vaccination strategies. For example, the joint WHO/FAO/OIE meetings on avian influenza strain H5N1 HPAI have recommended that all vaccination is practiced using a DIVA, so spread of infection can be monitored. However, current DIVA methods are difficult to scale-up and often have problems with the differentiation of vaccination from infection with other circulating viral strains.

[0008] Current methods of monitoring include physical tagging of vaccinated animals, the use of sentinel animals, and virological testing. However, these current methods have a number of limitations due to logistical and economic reasons.

[0009] The physical tagging of vaccinated animals involves the time consuming individual identification of vaccinated individuals by physical means such as ear tags, leg bands or wing tags. Also, the use of unvaccinated sentinel animals is logistically and economically difficult and there is also a risk that if sentinels become infected with the virus, e.g. poultry infected with H5N1 virus, there is increased risk of spread to humans. Virological testing of individuals via screening and detection of live virus or RT-PCR surveillance testing is a very expensive and infrastructure heavy process, which is not applicable for subunit

vaccines, and only provides information relating to the current status of an individual, and does not allow analysis of the infection and/or vaccination history of that individual.

[0010] In view of said limitations, the use of marker vaccines allowing a serological discrimination of vaccinated and infected animals is highly preferable, wherein such marker vaccines can be prepared either as negative or positive marker vaccine.

[0011] A negative marker vaccine is prepared by using an antigenic portion of the pathogen or by the removal of an antigen from the pathogen, which provokes specific antibodies in infected animals. Negative marker vaccines are usually either subunit vaccines or attenuated live vaccines containing a genetically engineered strain lacking an immunogenic antigen. An example for a negative marker vaccine is e.g. the use of baculovirus-expressed classical swine fever virus (CSFV) E2 protein as a subunit antigen for vaccinating against classical swine fever, wherein a detection of antibodies specific for other antigens of CSFV, e.g. E^{RNS} protein or NS3 protein, in sera of vaccinated pigs shows a CSFV infection.

[0012] A positive marker vaccine contains an additional antigen which induces specific antibodies in vaccinated individuals but not in infected ones. An example for a positive marker vaccine approach is described in WO 2007/053899 A1, where inactivated H6N2 Avian Influenza (AI) virus and tetanus toxin, both of which separately produced, were combined in one injection for vaccinating birds, and subsequently antibodies specific for tetanus toxin were detected in sera obtained from said birds as markers showing that the birds were vaccinated.

[0013] However, the separate production of both the vaccine antigen and the marker antigen is relatively expensive and, furthermore, a mixing step is required for combining both components in one vaccinating agent, wherein this additionally may also affect the stability of the vaccine components/antigens.

[0014] Thus, a simple marker system is needed for inexpensively producing positive marker vaccines, in particular subunit vaccines comprising baculovirus-expressed antigens.

[0015] Furthermore, effective compliance markers are needed which also enable the sensitive identification of vaccinations with a low amount of baculovirus-expressed subunit antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1: Amplified products were run on a gel to verify size.

[0017] FIG. 2: Cut insert and vector were run on a gel to check for linearization of vector.

[0018] FIG. 3: Positive clones from wells 2, 5, 7, 10, 11, and 12 were sequenced and the contigs were aligned with the construct reference sequence to check for mutations; all clones were a perfect match.

[0019] FIG. 4: Western blots to evaluate expression of G gene construct.

[0020] FIG. 5: The supernatant, soluble and insoluble cell fractions were probed for the protein; at this time protein is present in the insoluble portion.

[0021] FIG. 6: Results of size exclusion chromatography run using isocratic conditions on an AKTA. Elution of proteins from the column was monitored with UV absorption at 280 nm.

[0022] FIG. 7: Real Time PCR results: Column 1 indicates the well numbers. Column 2 shows the fluorophore_6-carboxyfluorescein (FAM) linked to the specific probe used. Column 3 indicates the fractions of SfRV antigen derived from size exclusion chromatography (fractions A11, A12, B1, B5, B12 and C6) or the standards with known quantities of SfRV specific nucleic acid used to generate the standard curve (wells 7 -14). Well 15 is the negative control (no template) and well 16 is the positive control containing concentrated SfRV antigen prior to fractionation by seize (SEC).

[0023] FIG. 8: ELISA; ELISA plates were coated with four different antigens including semi-purified SfRV (panel A), size exclusion fractions A11 (Panel B), A12 (Panel C) and B1 (Panel D). Plates were probed with sera from negative control animals (inverted triangles) or Day 28 sera from animals administered with experimental vaccine containing SfRV (circles).

DESCRIPTION OF THE INVENTION

[0024] The solution to the above technical problems is achieved by the description and the embodiments characterized in the claims.

[0025] Thus, the invention in its different aspects is implemented according to the claims.

[0026] The invention is based on the surprising finding that the use of Sf+ cells, which are infected with a rhabdovirus, for producing baculovirus-expressed antigens enables the inexpensive and efficient production of positive marker vaccines, and an easy and production inherent compliance marking which allows a sensitive method showing the proper delivery of the subunit vaccine.

[0027] In a first aspect, the invention thus provides a method of determining whether an individual has received an immunogenic composition, in particular a vaccine, comprising a recombinant protein produced by an expression system, preferably by a baculovirus expression system, in cultured insect cells, wherein said method, comprises the steps of: obtaining a biological sample from an individual; and determining in said biological sample the presence or absence of one or more markers showing that the individual has received one or more antigens from a virus which is an RNA virus capable of infecting insect cells, wherein the presence of said one or more markers in said biological sample indicates that said individual has received said immunogenic composition or wherein the absence of said one or more markers in said biological sample indicates that said individual has not received said immunogenic composition.

[0028] The term "recombinant protein", as used herein, in particular refers to a protein molecule which is expressed from a recombinant DNA molecule, such as a polypeptide which is produced by recombinant DNA techniques. An example of such techniques includes the case when DNA encoding the expressed protein is inserted into a suitable expression vector, preferably a baculovirus expression vector, which is in turn used to transfect, or in case of a baculovirus expression vector to infect, a host cell to produce the protein or polypeptide encoded by the DNA. The term "recombinant protein", as used herein, thus in particular refers to a protein molecule which is expressed from a recombinant DNA molecule.

[0029] According to a particular example, the recombinant protein is produced by a method with the following steps: the gene encoding the protein is cloned into a baculovirus transfer vector; the transfer vector is used to prepare recombinant baculovirus containing said gene by homologous recombination in insect cells; and the protein is then expressed in insect cells during infection with the recombinant baculovirus.

[0030] According to an alternative example, the recombinant protein is expressed in insect cells from a recombinant expression plasmid. In the case of this alternative example baculovirus is not needed.

[0031] It is further understood that the term "recombinant protein consisting of a sequence" in particular also concerns any cotranslational and/or posttranslational modification or modifications of the sequence affected by the cell in which the polypeptide is expressed. Thus, the term "recombinant protein consisting of a sequence", as described herein, is also directed to the sequence having one or more modifications effected by the cell in which the polypeptide is expressed, in particular modifications of amino acid residues effected in the protein biosynthesis and/or protein processing, preferably selected from the group consisting of glycosylations, phosphorylations, and acetylations.

[0032] Preferably, the recombinant protein of the present invention is produced or obtainable by a baculovirus expression system, in particular in cultured insect cells.

[0033] The term “expression system” as used herein particularly includes vehicles or vectors for the expression of a gene in a host cell as well as vehicles or vectors which bring about stable integration of a gene into the host chromosome.

[0034] As used herein “baculovirus expression system” in particular means a system for producing a desired protein in an insect cell using a recombinant baculovirus vector designed to express said protein. A baculovirus expression system generally comprises all elements necessary to achieve recombinant protein expression in insect cells, and typically involves the engineering of a baculovirus vector to express a desired protein, the introduction of the engineered baculovirus vector into insect cells, the culturing of the insect cells containing the engineered baculovirus vector in a suitable growth medium such that the desired protein is expressed, and the recovery of the protein. Typically, engineering a baculovirus vector involves the construction and isolation of recombinant baculoviruses in which the coding sequence for a chosen gene is inserted behind the promoter for a nonessential viral gene, wherein most of the presently used baculovirus expression systems are based on the sequence of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) ((Virology 202 (2), 586-605 (1994), NCBI Accession No.: NC_001623). Baculovirus expression systems are well known in the art and have been described, for example, in “Baculovirus Expression Vectors: A Laboratory Manual” by David R. O'Reilly, Lois Miller, Verne Luckow, pub. by Oxford

Univ. Press (1994), "The Baculovirus Expression System: A Laboratory Guide" by Linda A. King, R. D. Possee, published by Chapman & Hall (1992). An exemplary non-limiting example of a baculovirus system for producing a recombinant protein is e.g. described in WO 2006/072065 A2.

[0035] According to said first aspect, the present invention hence provides a method of determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by an expression system in cultured insect cells, said method also being termed "the method of the present invention" hereinafter, wherein said method in particular comprises determining in a biological sample obtained from said individual the presence or absence of one or more markers showing that the individual has received one or more antigens from a virus which is an RNA virus capable of infecting insect cells, and wherein the presence of said one or more markers in said biological sample indicates that said individual has received said immunogenic composition.

[0036] "Insect cell" as used herein means a cell or cell culture derived from an insect species. Of particular interest with respect to the present invention are insect cells derived from the species Spodoptera frugiperda and Trichoplusia ni.

[0037] As used herein, a "virus capable of infecting insect cells" is particularly understood as a virus harboring structures on the viral surface that are capable of interacting with insect cells to such an extent that the virus, or at least the viral genome, becomes incorporated into the insect cell.

[0038] Said infection of an insect cell more particular includes attachment of the virus to a host cell, entry of the virus into the cell, uncoating of the virion in the cytoplasm, replication and transcription of the viral genome, expression of viral proteins and assembly and release of new infectious viral particles.

[0039] Preferably, the immunogenic composition of the present invention is a marker vaccine, in particular a positive marker vaccine.

[0040] The term "marker vaccine" as described herein, in particular specifies a vaccine leading to an immunization in the immunized organism, which differs from the immunization of the organism caused by the real pathogen.

[0041] A "positive marker vaccine" particularly relates to a marker vaccine containing an additional antigen which induces the production of specific antibodies present in vaccinated individuals but not in infected ones.

[0042] The term "marker" as used within the context of the present invention is preferably equivalent to the term "biomarker", and in particular refers to a measurable substance or compound which indicates that an individual has been exposed to an immunogenic composition, preferably to a positive marker vaccine or, more particular, to the additional antigen of a positive marker vaccine which induces the production of specific antibodies found in vaccinated subjects but not in infected ones.

[0043] As used herein, the term "immunogenic composition" in particular refers to a composition that will elicit an immune response in an individual that has been exposed to the composition. An immune response may include induction of antibodies and/or induction of a T-cell response. Depending on the intended function of the composition, one or more antigens may be included. Preferably, the immunogenic composition as described herein is a vaccine.

[0044] The term "vaccine" as used herein, is defined in accordance with the pertinent art and relates to a composition that induces or enhances immunity of an individual to a particular disease. To this end, the vaccine comprises a compound that is similar to the pathogen or a compound of said pathogen causing said disease. Upon contact with this compound, the immune system of the individual is triggered to recognize the compound as foreign and to destroy it. The immune system subsequently remembers the contact with this compound, so that at a later contact with the disease-causing pathogen an easy and efficient recognition and destruction of the pathogen is ensured. In accordance with the present invention, the vaccine may be in any formulation for vaccines known in the art, such as for example vaccines for intramuscular injection, mucosal vaccines or vaccines for subcutaneous or intradermal injection as well as vaccines for inhalation, such as e.g. as aerosols. Such vaccine formulations are well known in the art and have been described, e.g. in Neutra MR et al. 2006 Mucosal vaccines: the promise and the challenge 6(2): 148-58 or F. P. Nijkamp, Michael J. Parnham 2011; Principles of Immunopharmacology ISBN-13: 978-3034601351.

[0045] The method of the present invention is thus in particular a method of determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells,

wherein said method comprises determining in a biological sample obtained from said individual the presence or absence of one or more markers showing that the individual has received one or more antigens from a virus which is an RNA virus capable of infecting insect cells, and wherein the presence of said one or more markers in said biological sample indicates that said individual has received said immunogenic composition.

[0046] Preferably, the biological sample is obtained from said individual at least 14 days and most preferably 14 to 35 days after the day the individual has been vaccinated or, respectively, has been supposedly vaccinated.

[0047] Preferably, the insect cell, as mentioned herein, is a *Spodoptera Frugiperda* (Sf) cell or a cell from a cell line derived from *Spodoptera Frugiperda,* and is more preferably selected from the group consisting of Sf9 cell and Sf+ cell. Respectively, the insect cells, as mentioned herein, are preferably *Spodoptera Frugiperda* (Sf) cells or cells from a cell line derived from *Spodoptera Frugiperda,* and are more preferably selected from the group consisting of Sf9 cells and Sf+ cells.

[0048] The one or more markers showing that the individual has received one or more antigens from an RNA virus capable of infecting insect cells, as mentioned herein, which are also termed "the one or more markers of the present invention" hereinafter, are preferably one or more markers selected from the group consisting of: antibodies specific for one or more antigens from a virus which is an RNA virus capable of infecting insect cells; one or more antigens from a virus which is an RNA virus capable of infecting insect cells, and; one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells.

[0049] Most preferably, the one or more markers of the present invention are antibodies specific for an antigen from a virus which is an RNA virus capable of infecting insect cells.

[0050] Preferably, the antibodies as described herein are polyclonal antibodies.

[0051] As used herein, the term "antibodies specific for" a defined antigen in particular refers to antibodies, preferably polyclonal antibodies, that bind an antigen with an affinity or K_a (i.e., an equilibrium association constant of a particular binding interaction with units of 1/M) of, for example, greater than or equal to about $10^5\ M^{-1}$, $10^6\ M^{-1}$, $10^7\ M^{-1}$, $10^8\ M^{-1}$, $10^9\ M^{-1}$, $10^{10}\ M^{-1}$, $10^{11}\ M^{-1}$, $10^{12}\ M^{-1}$ or $10^{13}\ M^{-1}$. Alternatively, binding affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M

(e.g., 10^{-5} M to 10^{-13} M). Binding affinities of antibodies can be readily determined using techniques well known to those of skill in the art (see, e.g., Scatchard *et al.* (1949) Ann. N.Y. Acad. Sci. 51:660; U.S. Pat. Nos. 5,283,173; 5,468,614; BIACORE® analysis; or the equivalent).

[0052] The one or more antigens from an RNA virus capable of infecting insect cells, as mentioned herein, which are also termed "the one or more antigens according to the present invention" hereinafter, is preferably a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:1 and/or a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7.

[0053] Regarding the term "at least 90%", as mentioned in the context of the present invention, it is understood that said term preferably relates to "at least 91%", more preferably to "at least 92%", still more preferably to "at least 93%" or in particular to "at least 94%".

[0054] Regarding the term "at least 95%" as mentioned in the context of the present invention, it is understood that said term preferably relates to "at least 96%", more preferably to "at least 97%", still more preferably to "at least 98%" or in particular to "at least 99%".

[0055] The term "having 100% sequence identity", as used herein, is understood to be equivalent to the term "being identical".

[0056] As used herein, the term "antigen" in particular refers to any molecule, moiety or entity capable of eliciting an immune response. This includes cellular and/or humoral immune responses.

[0057] Percent sequence identity has an art recognized meaning and there are a number of methods to measure identity between two polypeptide or polynucleotide sequences. See, e.g., Lesk, Ed., *Computational Molecular Biology*, Oxford University Press, New York, (1988); Smith, Ed., *Biocomputing: Informatics And Genome Projects*, Academic Press, New York, (1993); Griffin & Griffin, Eds., *Computer Analysis Of Sequence Data, Part I*, Humana Press, New Jersey, (1994); von Heinje, *Sequence Analysis In Molecular Biology*, Academic Press, (1987); and Gribskov & Devereux, Eds., *Sequence Analysis Primer*, M Stockton Press,

New York, (1991). Methods for aligning polynucleotides or polypeptides are codified in computer programs, including the GCG program package (Devereux et al., *Nuc. Acids Res.* 12:387 (1984)), BLASTP, BLASTN, FASTA (Atschul et al., *J. Molec. Biol.* 215:403 (1990)), and Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) which uses the local homology algorithm of Smith and Waterman (*Adv. App. Math.,* 2:482-489 (1981)). For example, the computer program ALIGN which employs the FASTA algorithm can be used, with an affine gap search with a gap open penalty of −12 and a gap extension penalty of −2. For purposes of the present invention, nucleotide sequences are aligned using Clustal W method in MegAlign software version 11.1.0 (59), 419 by DNASTAR Inc. using the default multiple alignment parameters set in the program (Gap penalty =15.0, gap length penalty =6.66, delay divergent sequence (%)= 30%, DNA transition weight =0.50 and DNA weight matrix= IUB) and, respectively, protein/amino acid sequences are aligned using Clustal W method in MegAlign software software version 11.1.0 (59), 419 by DNASTAR Inc. using the default multiple alignment parameters set in the program (Gonnet series protein weight matrix with Gap penalty =10.0, gap length penalty =0.2, and delay divergent sequence (%)= 30%).

[0058] As used herein, it is in particular understood that the term “sequence identity with the sequence of SEQ ID NO:X” is equivalent to the term “sequence identity with the sequence of SEQ ID NO:X over the length of SEQ ID NO: X” or to the term “sequence identity with the sequence of SEQ ID NO:X over the whole length of SEQ ID NO: X”, respectively. In this context, “X” is any integer selected from 1 to 24 so that “SEQ ID NO: X” represents any of the SEQ ID NOs mentioned herein.

[0059] The one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells, as mentioned herein, which are also termed “the one or more nucleic acid molecules according to the present invention” hereinafter, is preferably a nucleic acid molecule which encodes: a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO: 1 and/or; a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7; and/or an RNA having a sequence that is inverse complementary to a nucleic acid sequence having at least 70%, preferably at least 80%, more

preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:9; and/or a sequence that is inverse complementary to a nucleic acid sequence having at least at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:15.

[0060] Preferably, the method of the present invention comprises the steps of: contacting the biological sample with a capture reagent immobilized to a solid support, wherein the immobilized capture reagent is capable of binding the one or more markers of the present invention; and determining the presence or absence of said one or more markers bound to the capture reagent, wherein the presence of said one or more markers bound to the capture reagent is indicative for the presence of said one or more markers in said biological sample.

[0061] The term "capture reagent", as used herein, in particular refers to a molecule or a multi-molecular complex that can bind to a marker. The capture reagent is preferably capable of binding the marker in a substantially specific manner, preferably with an affinity or $K_a > 10^5\ M^{-1}$ or preferably $> 10^6\ M^{-1}$. The capture reagent may optionally be a naturally occurring, recombinant, or synthetic biomolecule. Proteins and nucleic acid ligands (aptamers) are highly suitable as capture agents. A whole virus or a virus fragment or a synthetic peptide may also serve as preferred capture reagents, since they are capable of binding antibodies.

[0062] As used herein the term "immobilized" particularly means that the capture reagent can be attached to a surface (e.g., the solid support) in any manner or any method; including, e.g., reversible or non- reversible binding, covalent or non-covalent attachment, and the like.

[0063] The herein mentioned capture reagent being immobilized to a solid support and being capable of binding one or more markers of the present invention, wherein said capture reagent is also termed "capture reagent according to the present invention" hereinafter, is preferably selected from the group consisting of: a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 1 to 6; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still

more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; an RNA virus capable of infecting insect cells, wherein said virus optionally has been inactivated; an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:9; and an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:15.

[0064] The term “specific hybridization” as described herein in particular relates to hybridization under stringent conditions. Said hybridization conditions may be established according to conventional protocols described, for example, in Sambrook, “Molecular Cloning, A Laboratory Handbook”, 2nd edition (1989), CSH Press, Cold Spring Harbor, N. Y.; Ausubel, “Current Protocols in Molecular Biology”, Green Publishing Associates and Wiley Interscience, N.Y. (1989); or Higgins and Hames (eds) “Nucleic acid hybridization, a practical approach” IRL Press Oxford, Washington DC (1985). An example for specific hybridization conditions is hybridization in 4×SSC and 0.1% SDS at 65° C. with subsequent washing in 0.1×SSC, 0.1% SDS at 65° C. Alternatively, stringent hybridization conditions are, for example, 50% formamide, 4×SSC at 42° C.

[0065] The term "solid support", as mentioned herein, denotes a non-fluid substance, and includes chips, vessels, and particles (including microparticles and beads) made from materials such as polymer, metal (paramagnetic, ferromagnetic particles), glass, and ceramic; gel substances such as silica, alumina, and polymer gels; capillaries, which may be made of polymer, metal, glass, and/or ceramic; zeolites and other porous substances; electrodes; microtiter plates; solid strips; and cuvettes, tubes or other spectrometer sample containers. A solid support component of an assay is distinguished from inert solid surfaces with which the assay may be in contact in that a "solid support" contains at least one moiety on its surface, which is intended to interact with the capture reagent, either directly or indirectly. A solid support may be a stationary component, such as a tube, strip, cuvette, or microtiter plate, or may be non- stationary components, such as beads and microparticles. Microparticles can also be used as a solid support for homogeneous assay formats. A variety of microparticles that allow both non-covalent or covalent attachment of proteins and other substances may be used. Such particles include polymer particles such as polystyrene and poly(methylmethacrylate); gold particles such as gold nanoparticles and gold colloids; and ceramic particles such as silica, glass, and metal oxide particles. See for example Martin, C.R., et al., Analytical

Chemistry-News & Features 70 (1998) 322A-327A, which is incorporated herein by reference.

[0066] A "chip" is a solid, non-porous material, such as metal, glass or plastics. The material may optionally be coated, entirely or in certain areas. On the surface of the material any array of spots is present, either visible or in coordinates. On each spot a defined polypeptide, with or without linker or spacer to the surface of the material, may be immobilized. All documents mentioned herein, both supra and infra, are hereby incorporated herein by reference.

[0067] The RNA virus capable of infecting insect cells, as mentioned herein, which is also termed “the RNA virus according to the present invention” hereinafter, is preferably: a (-)ssRNA virus and is optionally a virus which belongs to the family *Rhabdoviridiae*; and/or a virus which comprises a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:1 and/or; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7; and/or a virus whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:1 and/or; a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7; and/or a virus whose genome comprises an RNA molecule having a sequence that is inverse complementary to a nucleic acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:9 and/or; a sequence that is inverse complementary to a nucleic acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:15.

[0068] All nucleotide sequences of the sequence listing are typed in 5’ - ‘3 direction. The sequences of SEQ ID NOs. 9 and 15 encode cDNAs having a positive polarity (+ strand).

The term "inverse complementary" means that the sequence is anti-parallel to the reference sequence.

[0069] The RNA virus according to the present invention is preferably able to replicate at least two or more preferably at least three weeks in an insect cell line.

[0070] Preferably, the method of the present invention comprises determining in the biological sample the presence or absence of the one or more markers of the present invention, wherein said markers are antibodies specific for a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:1 or; antibodies specific for a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7; and wherein said method comprises the steps of:

a. contacting the biological sample with a capture reagent immobilized to a solid support, wherein the capture reagent is selected from the group consisting of a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 1 to 6 or a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8, an optionally inactivated virus which comprises a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:1 and/or; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7, a virus whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity

with the sequence of SEQ ID NO:1 and/or; a protein comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7, wherein said virus optionally has been inactivated, a virus whose genome comprises an RNA molecule which comprises a sequence that is inverse complementary to a nucleic acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:9 and/or; a sequence that is inverse complementary to a nucleic acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:15, wherein said virus optionally has been inactivated.

b. separating the biological sample from the immobilized capture reagent;

c. contacting the immobilized capture reagent-antibody complex with a detectable agent that binds to the antibody of the reagent-antibody complex; and

d. measuring the level of antibody bound to the capture reagent using a detection means for the detectable agent, and wherein the measuring step (d) preferably further comprises a comparison with a standard curve to determine the level of antibody bound to the capture reagent.

[0071] Preferably, said detectable agent that binds to the antibody of the reagent-antibody complex is a detectable antibody, more preferably a labelled secondary antibody.

[0072] The capture reagent, as described herein, is preferably a baculovirus-expressed protein, and said baculovirus-expressed protein is preferably expressed by the baculovirus of the present invention, which is described herein underneath.

[0073] According to another preferred aspect of the invention, the one or more markers of the present invention may also be one or more T cells specific for the RNA virus according to the invention and/or one or more B cells specific for the RNA virus according to the invention and/or one or more antigen-presenting cells presenting one or more antigens according to the present invention. The presence or absence of said one or more B cells and/or

said one or more T cells and/or said one or more antigen-presenting cells is preferably determined by means of a flow cytometry analysis, and wherein in particular one or more fluorescence labeled antigens according to the present invention are used for labeling said one or more B cells and/or said one or more T cells and/or wherein one or more fluorescence labeled antibodies specific for the RNA virus according to the present invention are used for labeling said one or more antigen-presenting cells.

[0074] The recombinant protein produced by an expression system in cultured insect cells, as mentioned herein, which is also termed "recombinant protein of the present invention" hereinafter is preferably PCV2 ORF2 protein, and said PCV2 ORF2 protein is in particular a protein having at least 90%, preferably at least 91%, more preferably at least 92%, still more preferably at least 93% or in particular at least 94% or at least 95% sequence identity with the sequence of SEQ ID NO:23.

[0075] According to another preferred aspect the recombinant protein of the present invention is influenza hemagglutinin, in particular avian influenza hemagglutinin, wherein said avian influenza hemagglutinin is preferably H5 protein of H5N1 virus, and wherein said H5 protein of H5N1 virus is more preferably a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:24.

[0076] The method of the present invention preferably further comprises the step of determining in the biological sample the presence of one or more analytes selected from the group consisting of: antibodies specific for the recombinant protein of the present invention, a polypeptide specific for the recombinant protein of the present invention, a nucleotide sequence specific for the DNA sequence encoding the recombinant protein of the present invention.

[0077] Within the context of the method of the present invention, the immunogenic composition is preferably the immunogenic composition as described underneath.

[0078] The term "biological sample" as used herein refers to any sample that is taken from an individual (e.g. from a pig or a bird) and includes, without limitation, cell-containing bodily fluids, peripheral blood, blood plasma or serum, saliva, tissue homogenates, lung and other organ aspirates, and lavage and enema solutions, and any other source that is obtainable

from a human or animal subject. For animals, examples of a "biological sample" include blood, cells, feces, diarrhea, milk, mucus, phlegm, pus, saliva, semen, sweat, tear, urine, tears, ocular fluids, vaginal secretions, and vomit, if present in that animal.

[0079] The biological sample, as referred to herein, has preferably been isolated from a mammal or a bird, preferably from a pig or a chicken (Gallus gallus domesticus), and/or is particular selected from the group consisting of whole blood, blood plasma, serum, urine, and oral fluids. Herein, the term “serum” is meant to be equivalent to “blood serum”.

[0080] The term “oral fluids” as used herein, in particular refers to one or more fluids found in the oral cavity individually or in combination. These include, but are not limited to saliva and mucosal transudate. It is particularly understood that oral fluids can comprise a combination of fluids from a number of sources (e.g., parotid, submandibular, sublingual, accessory glands, gingival mucosa and buccal mucosa) and the term “oral fluids” includes the fluids from each of these sources individually, or in combination. The term “saliva” refers to a combination of oral fluids such as is typically found in the mouth, in particular after chewing. The term "mucosal transudate", as used herein, refers to fluid produced by the passive diffusion of serum components from oral mucosal interstitia into the oral cavity. Mucosal transudate often forms one component of saliva.

[0081] The immobilized capture reagent, as described herein, is preferably coated on a microtiter plate, in particular to a microtiter plate capable to be read out by an ELISA reader.

[0082] According to another aspect, the present invention provides a recombinant baculovirus, wherein said baculovirus comprises a DNA sequence encoding a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 1 to 6 and/or; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; and/or wherein said baculovirus comprises a DNA sequence comprising or consisting of a sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 9 to 14 and/or; a sequence having at least 70%, preferably at least 80%, more preferably at least

90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:15 or SEQ ID NO:16.

[0083] The present invention further provides a vector, in particular a transfer vector, which contains a DNA sequence encoding a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 1 to 6 and/or; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; and/or which contains a DNA sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NO: 9 to 14 and/or; a DNA sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:15 or SEQ ID NO:16.

[0084] The transfer vector within the context of the invention is preferably a "baculovirus transfer vector".

[0085] The term "transfer vector" is art-recognized and refers to a first nucleic acid molecule to which a second nucleic acid has been linked, and includes for example plasmids, cosmids or phages.

[0086] In certain embodiments, a transfer vector may be an "expression vector," which refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (i) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (ii) a DNA sequence encoding a desired protein which is transcribed into mRNA and translated into protein, and (iii) appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids," which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome.

The invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

[0087] Certain transfer vectors may contain regulatory elements for controlling transcription or translation, which may be generally derived from mammalian, microbial, viral or insect genes. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants, may additionally be incorporated.

[0088] Transfer vectors derived from viruses, which may be referred to as "viral vectors," may be employed in certain embodiments of the present invention. Some examples include baculoviruses, retroviruses, adenoviruses and the like. Viral vectors, in particular baculovirus vectors, e.g., a baculovirus transfer vector, are in particular preferred according to the present invention. As for expression vectors, viral vectors may include regulatory elements.

[0089] The design of any transfer vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers (e.g., ampicillin), may also be considered.

[0090] In still a further aspect the present invention provides an immunogenic composition, which is also termed "the immunogenic composition of the present invention" hereinafter, wherein said composition comprises a recombinant protein produced by a baculovirus expression system in cultured insect cells; and one or more antigens from the RNA virus according to the present invention, wherein said virus preferably has been inactivated; and wherein said recombinant protein is preferably selected from the group consisting of a PCV2 ORF2 protein preferably comprising or consisting of a sequence having at least 90%, preferably at least 91%, more preferably at least 92%, still more preferably at least 93% or in particular at least 94% or at least 95% sequence identity with the sequence of SEQ ID NO:23; and influenza hemagglutinin, in particular avian influenza hemagglutinin, preferably H5 protein of H5N1 virus, more preferably a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the

sequence of SEQ ID NO:24; and a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs:1 to 8.

[0091] The term “inactivated”, as used herein, means that the antigen does not cause disease, when administered to a mammalian host or does not replicate in a host cell.

[0092] Various physical and chemical methods of inactivation are known in the art. The term “inactivated” refers to a previously virulent or non-virulent virus has been irradiated (ultraviolet (UV), X-ray, electron beam or gamma radiation), heated, or chemically treated to inactivate, kill, while retaining its immunogenicity. In one embodiment, the inactivated virus disclosed herein is inactivated by treatment with an inactivating agent. Suitable inactivating agents include beta-propiolactone, binary or beta- or acetyl-ethyleneimine, glutaraldehyde, ozone, and Formalin (formaldehyde).

[0093] For inactivation by formalin or formaldehyde, formaldehyde is typically mixed with water and methyl alcohol to create formalin. The addition of methyl alcohol prevents degradation or cross reaction during the in activation process.

[0094] More particular, the term "inactivated" means that the virus is incapable of replication in vivo or in vitro. For example, the term “inactivated” may refer to a virus that has been propagated in vitro, e.g. and has then been deactivated using chemical or physical means so that it is no longer capable of replicating.

[0095] Preferably the virus according to the present invention which has been inactivated is a virus inactivated with binary ethyleneimine (BEI).

[0096] The present invention further provides a method of producing the immunogenic composition of the present invention, comprising the steps of introducing a recombinant baculovirus encoding said recombinant protein into an insect cell, wherein said insect cell is infected with said RNA virus capable of infecting insect cells, culturing said insect cell harboring said recombinant baculovirus and said RNA virus; and recovering said recombinant protein and said virus, preferably in the supernate; and preferably further comprises the initial step of inserting a DNA sequence encoding said recombinant protein into a transfer vector

capable of introducing said sequence into the genome of baculovirus, thereby producing recombinant baculovirus.

[0097] According to still another aspect, the present invention provides a kit, in particular a test kit, for determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells, wherein said kit contains one or more capture reagents immobilized to a solid support, wherein the one or more immobilized capture reagents are capable of binding of one or more markers selected from the group consisting of antibodies specific for one or more antigens according to the present invention; one or more antigens from an RNA virus according to the present invention; and one or more nucleic acid molecules according to the present invention; and wherein said one or more capture reagents are preferably selected from the group consisting of a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of any one of SEQ ID NOs: 1 to 6; a protein comprising or consisting of an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 90%, still more preferably at least 95% or in particular 100% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; an RNA virus capable of infecting insect cells, wherein said virus has been optionally inactivated, an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:9; and an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:15.

[0098] Furthermore, the present invention provides a primer or a pair of primers, respectively, selected from the group consisting of the sequences having at least 90% or preferably at least 95% sequence identity with the sequence of any one of SEQ ID NOs: 17 to 22.

[0099] According to another aspect, the capture reagent of the present invention comprises or consists of viral particles and/or virus like particles of an RNA virus capable of infecting insect cells, wherein said RNA virus capable of infecting insect cells is preferably the RNA virus according to the present invention, and wherein said capture reagent is obtainable by a method comprising the steps of

i) obtaining supernatant from a culture of insect cells infected with an RNA virus, preferably with the RNA virus of the present invention, wherein said supernatant comprises viral particles and/or virus like particles of the RNA virus, and wherein said insect cells are preferably not infected with a baculovirus and/or are preferably not transfected with a plasmid,

ii) separating cell debris from said viral particles and/or virus like particles via a separation step including a micro filtration through at least one filter, preferably two filters, wherein the at least one filter preferably having a pore size larger than said viral particles and/or virus like particles, in particular having a pore size of about 0.1 µm to about 4 µm, preferably of about 0.2 µm to about 2 µm, and collecting the filtrate,

iii) and optionally subjecting the filtrate of ii) which contains said viral particles and/or virus like particles to size exclusion chromatography, wherein preferably the presence of protein in the eluent is measured by measuring the absorbance of light of the eluent at 260 nm or 280 nm (A_{260} or A_{280}), and wherein the eluent exhibiting the first A_{260} or A_{280} peak is collected.

[0100] Also, the invention provides a composition comprising said capture reagent, wherein said composition is obtainable by said method.

[0101] In the size-exclusion chromatography (SEC) step described herein, molecules are separated according to size in a bed packed with an inert porous medium, especially an inert gel medium, which is preferably a composite of cross-linked polysaccharides, e.g., cross-linked agarose and dextran in the form of spherical beads. Molecules larger than the largest pores in the swollen gel beads do not enter the gel beads and therefore move through the chromatographic bed fastest. Smaller molecules, which enter the gel beads to varying extent depending on their size and shape, are retarded in their passage through the bed. Molecules are thus generally eluted in the order of decreasing molecular size. A SEC column comprising a medium appropriate for the size-exclusion chromatography described herein is in preferably the HiPrep 26/60 Sephacryl S300HR column (GE Healthcare Bio-Sciences).

[0102] It is in particular understood that the eluent exhibiting the first A_{260} or A_{280} peak is the fraction of the filtrate of ii) comprising the largest protein structures included in the filtrate of ii). Thus, the eluent exhibiting the first A_{260} or A_{280} peak is the eluent, or a portion

thereof, containing the majority of the viral particles and/or virus like particles included in the filtrate of ii).

EXAMPLES

[0103] The following examples are only intended to illustrate the present invention. They shall not limit the scope of the claims in any way.

Example 1:

Infection of Sf cells with a rhabdovirus, production of semi-purified rhabdovirus, and cloning and expression of rhabodovirus antigens

[0104] In order to confirm the infection of SF+ and Sf9 cells with a rhabdovirus, also termed SfRV or SFRV (Sf cell rhabdovirus) hereinafter, primers were designed so as to amplify SFRV G and N genes with the goal of inserting unique 5' and 3' restriction sites. In addition, the 3' end primer was designed to add a tobacco etch virus (TEV) protease cleavage site followed by a 6X histidine tag. This was done to enable purification of the expressed protein on a nickel column using the His tag and then cleave off the His tag using the TEV protease to generate native G or N protein.

[0105] The sequences of the primers used for the G gene constructs (comprising the sequence of SEQ ID NO:1) are the sequences set forth in SEQ ID NOs: 17 and 18, the sequence of the nucleic acid for the G gene construct is provided in SEQ ID NO:12, and the amino acid sequence for the G gene construct is the sequence of SEQ ID NO:4.

[0106] The sequences of the primers used for the N gene constructs (comprising the sequence of SEQ ID NO:7) are the sequence set forth in SEQ ID NOs: 21 and 22, the sequence of the nucleic acid for the N gene construct is provided in SEQ ID NO:16, and the amino acid sequence for the N gene construct is the sequence of SEQ ID NO:8.

[0107] Further, transmembrane and intracellular domains of SFRV G glycoprotein were predicted using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) described in K. Hofmann & W. Stoffel (1993)TMbase - A database of membrane spanning proteins segments, Biol. Chem. Hoppe-Seyler 374,166, TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) which uses the hidden Markov model described in Möller S1, Croning MD, Apweiler R., Evaluation of methods for the prediction of membrane spanning regions,

Bioinformatics (2001) 17 (7): 646-653 and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/. Based on results from TMpred and TMHMM, the SFRV G sequence was terminated at amino acid 550 and the TEV cleavage site, 6X His tag and Pst I sites were added. The sequences of the primers usable for such G gene constructs (comprising the sequence of SEQ ID NO:2) are the sequences set forth in SEQ ID NOs: 17 and 19, the sequence of the nucleic acid for the G gene construct is provided in SEQ ID NO:13, and the amino acid sequence for the G gene construct is the sequence of SEQ ID NO:5.

[0108] Furthermore, the sequence of honey bee melittin secretory signal was fused to the sequence of a truncated SFRV G sequence (Chouljenko *et al.* J Virol, 84:8596–8606 (2010); Tessier *et al.* Gene. 98:177-83 (1991)), wherein the melittin sequence was added to full length SFRV G with TEV cleavage and 6X his by replacing its N terminus. The sequences of the primers usable for such G gene constructs (comprising the sequence of SEQ ID NO:3) are the sequences set forth in SEQ ID NOs: 20 and 18, the sequence of the nucleic acid for the G gene construct is provided in SEQ ID NO:14, and the amino acid sequence for the G gene construct is the sequence of SEQ ID NO:6.

[0109] The whole genome sequence of the SFRV according to MA *et al.* (J Virol. 88: 6576-6585 (2014)) deposited in GenBank (accession number KF947078) was used as the basis for primer design. Similarly, for TEV cleavage site the sequence ENLYFQG was used based on available published information.

[0110] SFRV was purified from the spent media used in the growth of Sf9 (adherent cells) and Sf+ (suspension cells): Spent media was collected from SFRV infected and conventionally propagated Sf9 and Sf+ cells and filtered through a 0.2 micron filter to eliminate cell debris. The filtrate was then loaded on to 30% sucrose cushions in NaCl-Tris HCl-EDTA (NTE) buffer pH 7.4 and subject to ultracentrifugation at 32,000 rpm at 4°C for 3 hours. The supernatant was carefully aspirated out and the pellet was rehydrated and re-suspended in NTE buffer. The total protein content was measured on a nanodrop machine and aliquots were assigned lot numbers and frozen at ≤-70°C till further use. This antigen preparation contained the semi-purified virus for coating ELISA plates, as described below.

[0111] The spent SF9 media was used as the source for SFRV viral RNA extraction. QIAamp viral RNA extraction kit (Qiagen) was used ad per manufacturer's instructions.

[0112] To amplify G and N genes, One-Step Superscript III kit was used as per manufacturer's instructions. A gradient RT-PCR was used with the following conditions: 1 cycle at 60°C for 30 minutes (RT step) followed by one cycle at 94°C for 2 minutes. This was followed by 40 cycles at 94°C for 15 seconds, annealing gradient 75°C-50°C for 60 seconds, followed by extension at 68°C for 2 minutes. Finally the reaction was subject to a single cycle at 68°C for 5 minutes and an infinite hold at 4°C.

[0113] Amplified products were run on a gel to verify size. Gel bands of expected size were cut out from gel and purified using Qiaquick gel extraction kit using manufacturer's instructions (**FIG. 1**).

[0114] In the following, only the further work using the G gene (comprising the sequence of SEQ ID NO:1) is described.

[0115] Amplified G gene (upper band in **FIG 1.**) with expected size of ~1.6Kb were cut out and gel extracted. as described earlier. This was then cut with Eco RI and Pst I restriction enzymes. The cut product is the insert. Similarly baculovirus transfer vector plasmid pVL1393 (Pharmingen) was cut with Eco RI and Pst I restriction enzymes to generate the vector. Cut insert and vector were run on a gel (see **FIG. 2**) to check for linearization of vector. The bands were cut out and gel extracted and the vector was dephosphorylated. Cloning the insert (Eco RI-PstI cut SFRV-G construct) into the vector (Eco RI-PstI cut pVL1393, dephosphorylated) and ligated using standard procedures.

[0116] Ligated product was used to transform E. coli cells (One Shot Max efficiency DH5α chemically competent cells from Invitrogen) and cells plated on LB Agar with Ampicillin. Colonies were picked up the next day and screened for uptake of plasmid using colony PCR and assigned clone numbers.

[0117] Reaction conditions for colony PCR were as follows: once cycle at 98°C for 3 minutes followed by 34 cycles of denaturation at 98°C for 30 seconds, Annealing at 58°C for 30 seconds and extension at 72°C for 2 minutes. This step was followed by a final extension step at 72°C for 10 minutes and a final hold at 4°C.

[0118] PCR products were run on an agarose gel to identify clones that contained the plasmid (see **FIG. 3**).

[0119] Positive clones were then grown on LB-Ampicillin broth and plasmid was purified from the cultures using Qiaprep miniprep plasmid purification kit (Qiagen) using manufacturer's instructions. The transfer vector thus generated contained the SFRV G gene construct and was aliquoted, assigned a lot number.

[0120] To generate recombinant baculoviruses expressing the SFRV G gene construct, the transfer plasmid was co-transfected along with linearized flashBAC ULTRAbaculovirus backbone DNA (Genway biotech) into Sf9 cells using ESCORT transfection reagent (SAFC). After a week, supernatants from the transfection (p0) were inoculated on to fresh Sf9 cells to amplify any recombinant baculoviruses that may have been generated.

[0121] The cell pellet from the transfection was collected and run on an SDS gel and transferred onto a nitrocellulose membrane for a Western blot. The protein was probed with anti His antibody (Invitrogen). The predicted protein size is ~71.5KDa. The gel shows (**FIG. 4**) two close bands at and above 62KDa marker for all five clones tested (2, 5, 7, 10 and 11) but no bands in cell control lane (cc).

[0122] Clones 2 and 5 of the recombinant virus was passed further and grown in spinner flasks to mass produce the protein in Sf+ suspension culture. The supernatant, soluble and insoluble cell fractions were probed for the protein. At this time protein was only present in the insoluble portion (**FIG. 5**). As a result, C-terminal truncations of SFRV G glycoproteins are being generated as a next step for use in an ELISA assay.

Example 2:

ELISA development

[0123] An ELISA was developed to evaluate the presence of an antibody response against SFRV in animals vaccinated with PCV2 or other subunit vaccines baculovirus expressed in SFRV infected Sf cells. Briefly, ELISA plates were coated with 250ng/well of semi-purified SFRV antigen (as described above) from either Sf9 or Sf+ cell supernatant.

- Coating was done by diluting the antigen in carbonate-bicarbonate buffer pH 9.0 so as to yield a final concentration of 250ng/well. Coating was done at 4°C overnight.
- Plates were washed the next day with PBS-Tween (PBST) and blocked with 10% milk for 1 hour at room temperature.

- Plates were then probed with 1:100 diluted serum (in blocking buffer) from animals vaccinated with PCV2 subunit antigen, baculovirus expressed in SFRV infected Sf cells, or unvaccinated controls (see data section).
- The plates were incubated at 37°C for 1 hour and then washed 5X times with PBST to eliminate unbound antibodies.
- Plates were then probed with 1:10,000 diluted secondary antibody (goat ant-pig IgG H+L-HRP conjugate – Bethyl laboratories), incubated 37°C for 1 hour and then washed 5X times with PBST to eliminate unbound antibodies.
- Finally, SureBlue TMB substrate (KPL) was added and plates were incubated for 5 minutes at room temperature and then stopped with TMB stop solution (KPL).
- Plates were then read at 450nm.

The ELISA plate set up was:

- Row a, wells 1-10 were coated with Sf9 derived SFRV
- Row b, wells 1-10 were coated with Sf+ derived SFRV
- Swine sera were evaluated in duplicates and animals vaccinated with PCV2 (A, B) subunit antigen are shown in bold. These should show a positive readout if the animals had encountered SFRV through vaccination and generated antibodies to SFRV.
- Negative controls are shown in italics (C and D)
- Columns 9 and 10 are buffer controls (no primary antibody)

[0124] The results of the ELISA are shown in **Table 1**.

[0125] Table 1:

	A		B		*C*		*D*			
	1	**2**	**3**	**4**	*5*	*6*	*7*	*8*	9	10
Row a	**0,774**	**0,626**	**0,217**	**0,215**	*0,058*	*0,098*	*0,095*	*0,091*	0,044	0,039
Row b	**1,857**	**0,909**	**1,556**	**1,028**	*0,993*	*0,554*	*0,104*	*0,103*	0,041	0,033

Table 1 shows data evaluating SfRV antigen derived from Sf9 cells (Row A) and Sf+ cells (Row B). Four sera samples were evaluated in duplicates. Columns A and B contained day 28 serum from animals vaccinated with experimental vaccine while columns C and D contained serum from negative control animals. The results indicate that both Sf9 and Sf+ cells contained SfRV and could be used as the virus antigen. Furthermore, the specific recognition of the antigen in vaccinated but not control animals point to the usefulness of SfRV as an inherent compliance marker.

Data interpretation:

- Based on the ELISA read out, animals vaccinated with PCV2 subunit antigen (Groups A and B) show a good response against semi-purified SFRV.
- The negative control animals (Groups C and D) do not show a reaction to the semi-purified SFRV.
- The results indicate the usefulness of SFRV for inherent compliance marking and for a DIVA approach.

Example 3:

ELISA using the SFRV antigen (wherein the antigen is a protein comprising the sequence of any one of SEQ ID NOs: 1 to 6, or wherein the antigen is purified or semipurified virus according to the present invention) described above

[0126] SCOPE: Test serum (or oral fluids) samples for the presence of antibodies to SFRV antigens

[0127] MATERIALS AND METHODS

A. Equipment

- ELISA washer
- ELISA reader
- WFI for cell culture, USP (Gibco, catalog# A12873-02)
- Carbonate-Bicarbonate buffer (pH9.6) tablets (Sigma, catalog# C3041-100CAP)
- 96 well immuno plates (round or flat bottom plates, Nunc Maxisorb)
- 12-channel pipettors, miscellaneous pipettors with range of 1 µL to 1 mL.
- pipette tips
- 37°C incubator
- 4°C refrigerator
- Vortexer
- plate lids (Thermo, catalog# AB-0752)
- S-block 2mL dilution blocks (Phenix catalog# M-1810S, or equivalent)
- reagent reservoirs
- timer

B. Reagents

1. Coating Buffer: Carbonate-Bicarbonate Buffer

- 100 mL of WFI
- 1 capsule of Carbonate-Bicarbonate Buffer
- Open capsule, dispense powder into WFI, mix until dissolved
- filter sterilize solution using a 0.2 µm filter
- store at 4°C
- Expiry: 1 week
- Needed per assay (4 plates): 50mL

2. 10X PBS:

- 1 package PBS concentrate, Fisher BP665-1
- qs to 1L with GenPur H_2O (or equivalent)
- store at room temperature
- Expiry: 1 year

3. Wash Buffer Solution: 0.05% Tween 20 in Dulbeccos PBS.

- 0.5 mL of Tween 20, Fisher BP337, or equivalent
- 100 mL of 10X D-PBS, pH 7.2-7.4
- q.s. to 1L with GenPur H_2O (or equivalent)
- pH to 7.2 ± 0.1
- store at room temperature
- Expiry: 6 months
- Needed per assay (4 plates): 2 Liters

4. PBST:

- 500mL 1X PBS pH 7.4 (Gibco, catalog# 10010-023)
- 0.3 mL of tween 20, Fisher BP337, or equivalent
- Store at room temperature
- Expiry: 6 months
- Needed per assay (4 plates): 100mL

5. Block Solution: 10% Non-fat dry milk in PBST solution.

- 20 g blotting grade block, Bio-Rad 170-6404, or equivalent
- 200 mL of PBST
- Store at 4°C
- Expiry: 0 days
- Needed per assay (4 plates): 200mL

6. SFRV Antigen:

- Uninfected SF or SF+ cell culture supernatant is filtered through a 0.2 micron filter (Thermo cat 456-0020). In this context, "uninfected SF or SF+ cell culture supernatant" means supernatant of SF or SF+ cells in culture, wherein said cells are not infected with baculovirus, but are infected with SFRV.

Furthermore, in the context of the cells described in the present disclosure, the term "SF " is equivalent to the term "Sf ", the term "SF+" is equivalent to the the term "Sf+ ", and the term "SF9" is equivalent to the term "Sf9", respectively.

- The filtrate is loaded on a 30% sucrose cushion and centrifuged at 28,000-34,000 rpm at 4°C for 2-4 hours
- After centrifugation, the supernatant is carefully aspirated out and the pellet is suspended in NaCl-Tris-EDTA buffer pH 7.4
- This is the semi-purified antigen.
- The protein concentration is estimated spectrophotometrically and the protein is aliquoted and frozen at -70°C until use

7. 2° Antibody: Goat anti-Pig IgG h+l HRP Conjugated. Bethyl Labs Cat. No. A100-105P, stored at 4°C ± 3.0°C.

8. Substrate: SureBlue TMB 1-Component Microwell Peroxidase Substrate. Kirkgaard and Perry Laboratories Cat No. 52-00-01 or equivalent. Substrate will be stored at 4°C ± 3.0°C, pre-incubated at 25°C ± 2.0°C, and used at 25°C ± 3.0°C.

9. Stop Solution: TMB Stop Solution. Kirkgaard and Perry Laboratories Cat No. 50-85-04 or equivalent. Stored at room temperature.

C. Procedure

1. Prepare Coating Buffer using recipe listed in B1 above.
2. Dilute SFRV antigen in coating butter to 250ng/well. Mix by inverting 10 times antigen. Coat with 250ng/100µl (i.e. 2.5µg/ml = 250ng/well).
3. Add 100µL of diluted SFRV antigen to all wells,
4. Seal the test plate(s) with plate lids and incubate overnight at 4°C, place on the bottom of the refrigerator to minimize disturbance.

Next Day

5. Prepare enough blocking solution for current assay only. Recommend 200mL Block for 4 plates. Store at 4°C temperature until needed.
6. Wash test plate(s) 5 times with wash buffer using the ultrawash plus microtiter plate washer, or equivalent.

7. A 100µL of blocking solution to all the wells of test plate(s). Cover the test plate(s) and incubate for 1.0 hour at 37°C ± 2.0°C.
8. During blocking incubation, dilute test serum samples 1:100 in block in s-block. For oral fluids, dilute the samples 1:2 in block. . Each sample tested individually. Dilute positive and negative controls in the same fashion. .
9. Wash test plate(s) 1X times. After last wash gently tap plates onto a paper towel.
10. Add 100µL per well of the pre-diluted test samples to respective plate(s). Avoid well contact with the tip of the pipette. Changing tips between each test sample.

    Cover test plate(s) and incubate for 1.0 hour at 37°C ± 2.0°C for serum samples. For oral fluids samples incubate for 16.0 hours at 4°C ± 2.0°C.
11. Just prior to washing the test plate(s), remove secondary antibody vial from refrigerator and dilute to 1:10,000 in block. Recommended to make serial dilutions to achieve a 1:10,000 dilution (4 dilutions). Mix diluted antibody by inversion 10 times.
12. Wash test plate(s) 5 times. .
13. Add 100µL of diluted detection antibody to all wells of the test plate(s). Cover test plate(s) and incubate for 1.0 hour at 37°C ± 2.0°C.
14. Immediately remove SureBlue TMB 1-Component Microwell Peroxidase Substrate from the refrigerator (4°C ± 3°C) and transfer appropriate volume to a brown, or opaque, High Density Polyethylene (HDPE) container, and incubate for 1 hour ± 15minutes at 25°C ± 2.0°C (bench top).
15. Wash test plate(s) 5 times. After last wash gently tap plates onto a paper towel. Recommend to turn on plate reader while plate(s) are washing.
16. Add 100µL of substrate to all wells of the test plate(s).
17. Incubate at 25°C ± 3°C for 5 minutes.
18. Stop the reaction with the addition of 100µL of stop solution to all wells
19. Measure absorbance at 450nm.

D. Acceptance Criteria / Results

- Positive control: serum (or oral fluid, respectively) from pigs hyper immunized with SFRV/SRFV G glycoprotein

- Naïve swine sera (or oral fluids, respectively) or sera (or oral fluids, respectively) from unvaccinated swine that shows negligible to no reaction to SFRV antigen

Example 4:

Production of semi-purified rhabdovirus, size exclusion chromatography (SEC), Real Time PCR, electron microscopy of SEC fractions, and ELISA

Production of semi-purified rhabdovirus:

[0128] Prior to loading onto the column, semi-purified rhabdovirus was produced in that cell culture supernatant (40 mL) of SfRV infected Sf+ insect cells that was concentrated from 5 liters down to 800 mL using hollow fiber filtration was filtered through a 1.2 µm syringe filter. The resulting filtrate is the "semi-purified rhabdovirus" according to this example.

Size exclusion chromatography (SEC):

[0129] Size exclusion chromatography was run using isocratic conditions on an AKTA Explorer with a HiPrep 26/60 Sephacryl S300HR column (GE Healthcare Bio-Sciences) at a flow rate of 1 mL/min. The column was equilibrated with 1.5 column volumes of buffer (1X phosphate buffered saline, pH 7.4, Gibco) followed by injection of the clarified sample (approximately 5% column volume) of the semi-purified rhabdovirus produced according to (i). Separation occurred at a flow rate of 1.0 mL/min over 1.5 column volumes of buffer, and fractions (8 mL) were collected from the time of injection through the entire separation step. Elution of proteins from the column was monitored with UV absorption at 280 nm (**FIG. 6**)

[0130] Fractions were analyzed by 4-12% SDS-PAGE (Thermo Fisher) following concentration of peak fractions using TCA/acetone precipitation. Briefly, 1 mL of each fraction was precipitated with TCA (200 µL) for 1 hr on ice. The samples were centrifuged for 2 min at 20,000 xg, and the supernatant was removed. Fractions were washed with 500µL of ice cold acetone and mixed by vortexing followed by centrifugation for 2 min at 20,000 xg. The centrifugation and acetone steps were repeated for a total of three acetone washes. The pellets were dried for 20 min, suspended in 20µL of gel loading buffer, and loaded onto the gel. Gels were stained for 1 hr using Imperial protein stain (Thermo Fisher) and destained for at least 3 hr with deionized water. Following gel analysis, protein concentrations of

fractions were determined by BCA assay (Thermo Fisher) using bovine serum albumin as a standard.

Real Time PCR

The presence of SfRV RNA in the semi-purified rhabdovirus (filtrate) of (i) and in the fractions collected by the SEC of (ii) was detected/quantified by using the following methods and sequences for Real Time PCR:

Primers/Probes/G-block control:

Name	Sequence	Genomic Position*
Rhab_qPCR-F	SEQ ID NO:25	5584-5603
Rhab_qPCR-R	SEQ ID NO:26	5654-5672 (RC)
Rhab_qPCR-PR (FAM)	SEQ ID NO:27	5624-5646 (RC)
Rhab_gBlock	SEQ ID NO:28	5565-5690

* Genomic position based upon GenBank Reference strain: KF947078. All sequences target the region encoding the SfRV glycoprotein

Cycle conditions:

1 cycle @ 50°C for 10 min

1 cycle @ 95°C for 3 min

40 cycles @ 95°C for 15 sec.

57°C for 15 sec**Data collection (FAM)

Brief description of steps performed:

[0131] Amplification is performed using BioRad iTaq Universal Probes One-Step Kit (Cat # 172-5141) according to suggested manufacturers suggested protocol. Primers are added to a final concentration of 0.4µM in a 25µl reaction while probe is added to a final concentration of 0.16µM. In each run a standard curve composed a synthetic double-stranded

g-block (IDT) sequence corresponding to the expected amplicon. The reaction took place using a CFX96 real-time PCR detection system (BioRad) under the following conditions: initial reverse transcription at 50°C for 10min, followed by initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15s and annealing and extension at 57°C for 15s with data collection in FAM channel. The optical data were analyzed using CFX Manager software (version 2.1,BioRad). Runs were deemed valid based on: consistency of standard curve, r-squared values exceeding 0.99 and calculated efficiencies between 80-120%. For each determination, the threshold lines were automatically calculated using the regression setting for cycle threshold (Ct) determination mode. Baseline subtraction was done automatically using the baseline subtracted mode.

[0132] The results of the Real Time PCR are shown in **FIG. 7**.

In FIG. 7:

[0133] Column 1 indicates the well numbers. Column 2 shows the fluorophore_6-carboxyfluorescein (FAM) linked to the specific probe used in this realtime PCR. Column 3 indicates the fractions of SfRV antigen derived from size exclusion chromatography (fractions A11, A12, B1, B5, B12 and C6) or the standards with known quantities of SfRV specific nucleic acid used to generate the standard curve (wells 7 -14). Well 15 served as the negative control (no template) and well 16 served as the positive control containing concentrated SfRV antigen prior to fractionation by seize (SEC).

[0134] The quantitation cycle (Cq) is the cycle at which fluorescence is detected. Lower Cq values indicate higher copy numbers of the specific target in the sample. This data is shown in column 4. The extrapolated genomic copy numbers are shown in column 5 as sequence quantification (SQ) and shows the number of genomic copies per mL.

[0135] The data shows that the starting material had 6 logs of SfRV specific genomic copies/mL (well 16), similarly fractions A11 and A12 had 6 logs of genomic copies/mL. These two fractions along with tail end fraction B1 should contain the majority SfRV viral particles/virions and virus like particles (VLPs). The other fractions B5, B12 and C6 should contain subviral particles in SEC and therefore lower amounts of viral RNA if any.

Electron Microscopy:

[0136] Fractions collected by the SEC of (ii) were stained with 2.5% phospho tungstic acid (PTA) for 3 minutes (negative staining) for electron microscopy. In the fractions A11 and A12 (c.f. **FIG. 6**) particles of ~30-35 nm were observed, which were considered to be viral particles of SfRV.

ELISA:

[0137] The ELISA was performed as described in Example 2, wherein the materials and methods described under Example 3 were used, with the difference that instead of the "SFRV Antigen" (under point B. **6.** of Example 3) the semi-purified rhabdovirus (filtrate) of (i) and the fractions A11, A12, and B1 collected by the SEC of (ii) were used, each diluted in coating buffer to a concentration of 250 ng in 100 µl, and then 100 µl of each of said antigens was coated on a well.

[0138] As test serum samples, blood serum from animals immunized with an experimental vaccine was used, said vaccine comprising recombinant protein produced by a baculovirus expression system in cultured SfRV infected insect cells. The sera were obtained from blood taken from the animals 28 days after the administration of the experimental vaccine.

[0139] As negative control, blood serum of corresponding non-immunized animals was used, respectively.

[0140] The results of the ELISA are shown in **FIG. 8**.

[0141] ELISA plates were coated with four different antigens including semi-purified SfRV (panel A), size exclusion fractions A11 (Panel B), A12 (Panel C) and B1 (Panel D). Plates were probed with sera from negative control animals (inverted triangles) or Day 28 sera from animals administered with experimental vaccine containing SfRV (circles).

[0142] Results show that sera from vaccinated animal reacted to the coated antigens while the negative control serum had minimal reaction. Furthermore, vaccinated animals reacted strongly to wells coated with fractions A11, A12 and B1 (panels B, C and D) as evidenced by the increased OD values and reactions were more tightly clustered with these

fractions as compared to semipurified SfRV (panel A). This indicates a stronger recognition and more specific response to the coated antigen (fractions A11, A12 and B1).

In the sequence listing:

[0143] SEQ ID NO:1 corresponds to the sequence of a SFRV G protein,

[0144] SEQ ID NO:2 corresponds to the sequence of a truncated SFRV G protein,

[0145] SEQ ID NO:3 corresponds to the sequence of a truncated SFRV G protein with N-terminal melittin sequence,

[0146] SEQ ID NO:4 corresponds to SEQ ID NO:1 with modifications (including 6x His tag),

[0147] SEQ ID NO:5 corresponds to SEQ ID NO:2 with modifications (including 6x His tag),

[0148] SEQ ID NO:6 corresponds to SEQ ID NO:3 with modifications (including 6x His tag),

[0149] SEQ ID NO:7 corresponds to the sequence of a SFRV N protein,

[0150] SEQ ID NO:8 corresponds to SEQ ID NO:7 with modifications (including 6x His tag),

[0151] SEQ ID NO:9 corresponds to a sequence encoding SEQ ID NO:1,

[0152] SEQ ID NO:10 corresponds to a sequence encoding SEQ ID NO:2,

[0153] SEQ ID NO:11 corresponds to a sequence encoding SEQ ID NO:3,

[0154] SEQ ID NO:12 corresponds to a sequence encoding SEQ ID NO:4,

[0155] SEQ ID NO:13 corresponds to a sequence encoding SEQ ID NO:5,

[0156] SEQ ID NO:14 corresponds to a sequence encoding SEQ ID NO:6,

[0157] SEQ ID NO:15 corresponds to a sequence encoding SEQ ID NO:7,

[0158] SEQ ID NO:16 corresponds to a sequence encoding SEQ ID NO:8,

[0159] SEQ ID NO:17 corresponds to a forward primer to construct SEQ ID NO:12 or SEQ ID NO:13,

[0160] SEQ ID NO:18 corresponds to a reverse primer to construct SEQ ID NO:12 or SEQ ID NO:14,

[0161] SEQ ID NO:19 corresponds to a reverse primer to construct SEQ ID NO:13,

[0162] SEQ ID NO:20 corresponds to a forward primer to construct SEQ ID NO:14,

[0163] SEQ ID NO:21 corresponds to a forward primer to construct SEQ ID NO:16,

[0164] SEQ ID NO:22 corresponds to a reverse primer to construct SEQ ID NO:16,

[0165] SEQ ID NO:23 corresponds to a sequence of a PCV2 ORF2 protein,

[0166] SEQ ID NO:24 corresponds to a sequence of a hemagglutinin H5 protein (influenza virus).

CLAIMS

CLAIMS

1. A method of determining whether an individual has received an immunogenic composition comprising: a recombinant protein produced by an expression system in cultured insect cells, wherein said method comprises determining in a biological sample obtained from said individual the presence or absence of one or more markers showing that the individual has received one or more antigens from a virus which is an RNA virus capable of infecting insect cells, and wherein the presence of said one or more markers in said biological sample indicates that said individual has received said immunogenic composition.

2. The method of claim 1, wherein said expression system is a baculovirus expression system, and/or wherein said one or more markers showing that the individual has received one or more antigens from an RNA virus capable of infecting insect cells are one or more markers selected from the group consisting of:

   a. antibodies specific for one or more antigens from a virus which is an RNA virus capable of infecting insect cells;

   b. one or more antigens from a virus which is an RNA virus capable of infecting insect cells; and

   c. one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells.

3. The method of claim 1 or 2, wherein said one or more antigens from an RNA virus capable of infecting insect cells is a protein comprising or consisting of:

   a. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

   b. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7.

4. The method of claim 2 or 3, wherein said one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells is a nucleic acid molecule which encodes a protein comprising or consisting of:

a. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO: 1; and/or

b. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

c. said one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells is an RNA having a sequence that is inverse complementary to a nucleic acid sequence having at least at least 70% sequence identity with the sequence of SEQ ID NO:9; and/or

d. a sequence that is inverse complementary to a nucleic acid sequence having at least at least 70% sequence identity with the sequence of SEQ ID NO:15.

5. The method of any one of claims 1 to 4 comprising the steps of:

a. contacting the biological sample with a capture reagent immobilized to a solid support, wherein the immobilized capture reagent is capable of binding said one or more markers; and

b. determining the presence or absence of said one or more markers bound to the capture reagent, wherein the presence of said one or more markers bound to the capture reagent is indicative for the presence of said one or more markers in said biological sample.

6. The method of claim 5, wherein said capture reagent is selected from the group consisting of:

a. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with a sequence selected from the group consisting of any one of SEQ ID NOs: 1 to 6;

b. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8;

c. an RNA virus capable of infecting insect cells, wherein said virus has optionally been inactivated and/or wherein said virus is optionally semipurified whole virus;

d. an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:9; and

e. an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:15.

7. The method of any one of claims 1 to 6, wherein said RNA virus is

a. a (-)ssRNA virus, preferably a virus which belongs to the family *Rhabdoviridiae*; and/or

b. said RNA virus capable of infecting insect cells is a virus which comprises:

i. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

iii. said RNA virus capable of infecting insect cells is a virus whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

iv. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

v. said RNA virus capable of infecting insect cells is a virus whose genome comprises an RNA molecule having a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:9;

vi. and/or a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15.

8. The method of any one of claims 1 to 7, wherein said method comprises:

a. determining in said biological sample the presence or absence of said one or more markers, wherein said markers are antibodies specific for a protein comprising or consisting of:

i. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; or

ii. antibodies specific for a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and wherein said method comprises the steps of:

b. contacting the biological sample with a capture reagent immobilized to a solid support, wherein the capture reagent is selected from the group consisting of:

i. a protein comprising or consisting of a sequence having at least 70% sequence identity with a sequence selected from the group consisting of any one of SEQ ID NOs: 1 to 6, or a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8,

ii. an optionally inactivated virus which comprises a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1;

iii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

iv. a virus whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

v. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7, wherein said virus has optionally been inactivated,

vi. a virus whose genome comprises an RNA molecule which comprises a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:9; and/or

vii. a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15, wherein said virus has optionally been inactivated; and/or

viii. a synthetic peptide having a sequence selected from the sequences consisting of 5 to 11 consecutive amino residues of SEQ ID NO:1 or SEQ ID NO:7; and

c. separating the biological sample from the immobilized capture reagent;

d. contacting the immobilized capture reagent-antibody complex with a detectable agent that binds to the antibody of the reagent-antibody complex; and

e. measuring the level of antibody bound to the capture reagent using a detection means for the detectable agent.

9. The method of claim 8, wherein the measuring step (d) further comprises a comparison with a standard curve to determine the level of antibody bound to the capture reagent.

10. The method of claim 8 or 9, wherein said detectable agent that binds to the antibody of the reagent-antibody complex is a detectable antibody, preferably a labelled secondary antibody.

11. The method of any one of claims 5 to 10, wherein said capture reagent is a baculovirus-expressed protein, and wherein said baculovirus-expressed protein is preferably expressed by the baculovirus of claim 18.

12. The method of any one of claims 1 to 11 further comprising the step of determining in said biological sample the presence of one or more analytes selected from the group

consisting of antibodies specific for said recombinant protein, a polypeptide specific for said recombinant protein, a nucleotide sequence specific for the DNA sequence encoding said recombinant protein.

13. The method of any one of claims 1 to 12, wherein said recombinant protein is selected from the group consisting of PCV2 ORF2 protein, wherein said PCV2 ORF2 protein is preferably a protein having at least 90% sequence identity with the sequence of SEQ ID NO:23, and influenza hemagglutinin, preferably avian influenza hemagglutinin, in particular H5 protein of H5N1 virus, wherein said avian influenza hemagglutinin or H5 protein of H5N1 virus is preferably a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:24.

14. The method of any one of claims 1 to 13, wherein said immunogenic composition is the composition of any one of claims 20 to 23.

15. The method of any one of claims 1 to 14, wherein the biological sample has been isolated from a mammal or a bird, preferably from a pig or a chicken.

16. The method of any one of claims 1 to 15, wherein the biological sample is selected from the group consisting of whole blood, blood plasma, serum, urine, and oral fluids.

17. The method of any one of claims 1 to 16, wherein the immobilized capture reagent is coated on a microtiter plate.

18. A recombinant baculovirus, wherein said baculovirus comprises a DNA sequence encoding a protein comprising or consisting of:

a. an amino acid sequence having at least 70% sequence identity with a sequence selected from the group consisting of:

i. SEQ ID NOs: 1 to 6; and/or

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; and/or

b. wherein said baculovirus comprises a DNA sequence comprising or consisting of:

i. a sequence having at least 70% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 9 to 14, and/or

ii. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15 or SEQ ID NO:16.

19. A vector, in particular a transfer vector, which contains a DNA sequence encoding a protein comprising or consisting of:

a. an amino acid sequence having at least 70% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 1 to:6; and/or

b. a protein comprising or consisting of:

i. an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8; and/or

c. which contains a DNA sequence having at least 70% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 9 to 14; and/or a DNA sequence having at least 70% sequence identity with the sequence of SEQ ID NO: 15 or SEQ ID NO:16.

20. Immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells, and one or more antigens from a virus which is an RNA virus capable of infecting insect cells.

21. The immunogenic composition of claim 20 or 21, wherein said RNA virus capable of infecting insect cells has been inactivated.

22. The immunogenic composition of claim 20 or 21, wherein said recombinant protein is selected from the group consisting of:

a. a PCV2 ORF2 protein preferably comprising or consisting of a sequence having at least 90% sequence identity with the sequence of SEQ ID NO:23,

b. influenza hemagglutinin, preferably avian influenza hemagglutinin, in particular a H5 protein of H5N1 virus, wherein said avian influenza hemagglutinin or H5 protein of H5N1 virus is preferably a protein comprising or consisting of:

i. an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:24; and

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 1 to 8.

23. The immunogenic composition of any one of claims 20 to 22, wherein said RNA virus comprises:

a. a (-)ssRNA virus, preferably a virus which belongs to the family *Rhabdoviridiae*; and/or said RNA virus capable of infecting insect cells is a virus which comprises a protein comprising or consisting of:

i. an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

b. a RNA virus capable of infecting insect cells and whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of:

i. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

c. a RNA virus capable of infecting insect cells whose genome comprises:

i. an RNA molecule having a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:9; and/or

ii. a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15.

24. A method of producing the immunogenic composition of any one of claims 20 to 23, comprising the steps of:

a. introducing a recombinant baculovirus encoding said recombinant protein into an insect cell, wherein said insect cell is infected with said RNA virus capable of infecting insect cells culturing said insect cell harboring said recombinant baculovirus and said RNA virus; and

b. recovering said recombinant protein and said virus, preferably in the supernate.

25. The method of claim 24, further comprising the step of inserting a DNA sequence encoding said recombinant protein into a transfer vector capable of introducing said sequence into the genome of baculovirus, thereby producing recombinant baculovirus.

26. A kit for determining whether an individual has received an immunogenic composition comprising a recombinant protein produced by a baculovirus expression system in cultured insect cells, wherein said kit contains one or more capture reagents immobilized to a solid support, wherein the one or more immobilized capture reagents are capable of binding of one or more markers selected from the group consisting of:

a. antibodies specific for one or more antigens from a virus which is an RNA virus capable of infecting insect cells;

b. one or more antigens from a virus which is an RNA virus capable of infecting insect cells; and

c. one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells.

27. The kit of claim 26, wherein said one or more capture reagents are selected from the group consisting of:

a. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 1 to 6;

b. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7 or SEQ ID NO:8;

c. an RNA virus capable of infecting insect cells, wherein said virus has been inactivated, an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:9; and

d. an oligonucleotide that is capable of specific hybridization with sequences characteristic of the sequence SEQ ID NO:15.

28. The kit of any one of claims, wherein said RNA virus comprises or consists of:

a. a (-)ssRNA virus, preferably a virus which belongs to the family Rhabdoviridiae; and/or

b. a RNA virus capable of infecting insect cells which comprises a protein comprising or consisting of:

i. an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

c. a RNA virus capable of infecting insect cells whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of:

i. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

d. a RNA virus capable of infecting insect cells whose genome comprises:

i. an RNA molecule having a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:9; and/or

ii. a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15.

29. The kit of any one of claims 26 to 28, wherein said one or more antigens from an RNA virus capable of infecting insect cells is a protein comprising or consisting of:

a. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

b. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

c. one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells wherein said one or more nucleic acid molecules encode a protein comprising or consisting of:

i. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO: 1; and/or

ii. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

d. one or more nucleic acid molecules specific for an RNA virus capable of infecting insect cells wherein said one nucleic acid molecules is an RNA encoding a sequence comprising :

i. an inverse complement to a nucleic acid sequence having at least at least 70% sequence identity with the sequence of SEQ ID NO:3; and/or a

ii. an inverse complement to a nucleic acid sequence having at least at least 70% sequence identity with the sequence of SEQ ID NO:4.

30. The method of any one of claims 1 to 17, the immunogenic composition of any one of claims 20 to 23, the method of any one claim 24 or 25, or the kit of any one of claims 26 to 29, wherein said insect cell or insect cells are *Spodoptera Frugiperda* (Sf) cells or cells from a cell line derived from *Spodoptera Frugiperda*, preferably selected from the group consisting of Sf9 cells and Sf+ cells.

31. A composition comprising a capture reagent, the method of any one of claims 5 to 17 or 30, or the kit of any one of claims 26 to 30,

wherein said capture reagent comprises or consists of viral particles and/or virus like particles of an RNA virus capable of infecting insect cells, and wherein said composition or said capture reagent is obtainable by a method comprising the steps of

i) obtaining supernatant from a culture of insect cells infected with an RNA virus capable of infecting insect cells, wherein said supernatant comprises viral particles and/or virus like particles of the RNA virus, and wherein said insect cells infected with an RNA virus capable of infecting insect cells are preferably not infected with a baculovirus and/or are preferably not transfected with a plasmid,

ii) separating cell debris from said viral particles and/or virus like particles via a separation step including a micro filtration through at least one filter, preferably two filters, wherein the at least one filter preferably having a pore size larger than said viral particles and/or virus like particles, in particular having a pore size of about 0.1 µm to about 4 µm, preferably of about 0.2 µm to about 2 µm,

iii) and optionally subjecting the filtrate of ii) which contains said viral particles and/or virus like particles to size exclusion chromatography, wherein the presence of protein in the eluent is measured by measuring the absorbance of light at 260 nm or 280 nm (A_{260} or A_{280}), and wherein preferably the eluent exhibiting the first A_{260} or A_{280} peak is collected.

32. The composition, the kit, or the method of claim 31, wherein said RNA virus comprises or consists of:

a. a (-)ssRNA virus, preferably a virus which belongs to the family Rhabdoviridiae; and/or

b. an RNA virus capable of infecting insect cells which comprises a protein comprising or consisting of:

i. an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of an amino acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

c. an RNA virus capable of infecting insect cells whose genome comprises a nucleic acid molecule which encodes a protein comprising or consisting of:

i. a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:1; and/or

ii. a protein comprising or consisting of a sequence having at least 70% sequence identity with the sequence of SEQ ID NO:7; and/or

d. an RNA virus capable of infecting insect cells whose genome comprises:

i. an RNA molecule having a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:9; and/or

ii. a sequence that is inverse complementary to a nucleic acid sequence having at least 70% sequence identity with the sequence of SEQ ID NO:15.

FIG. 1

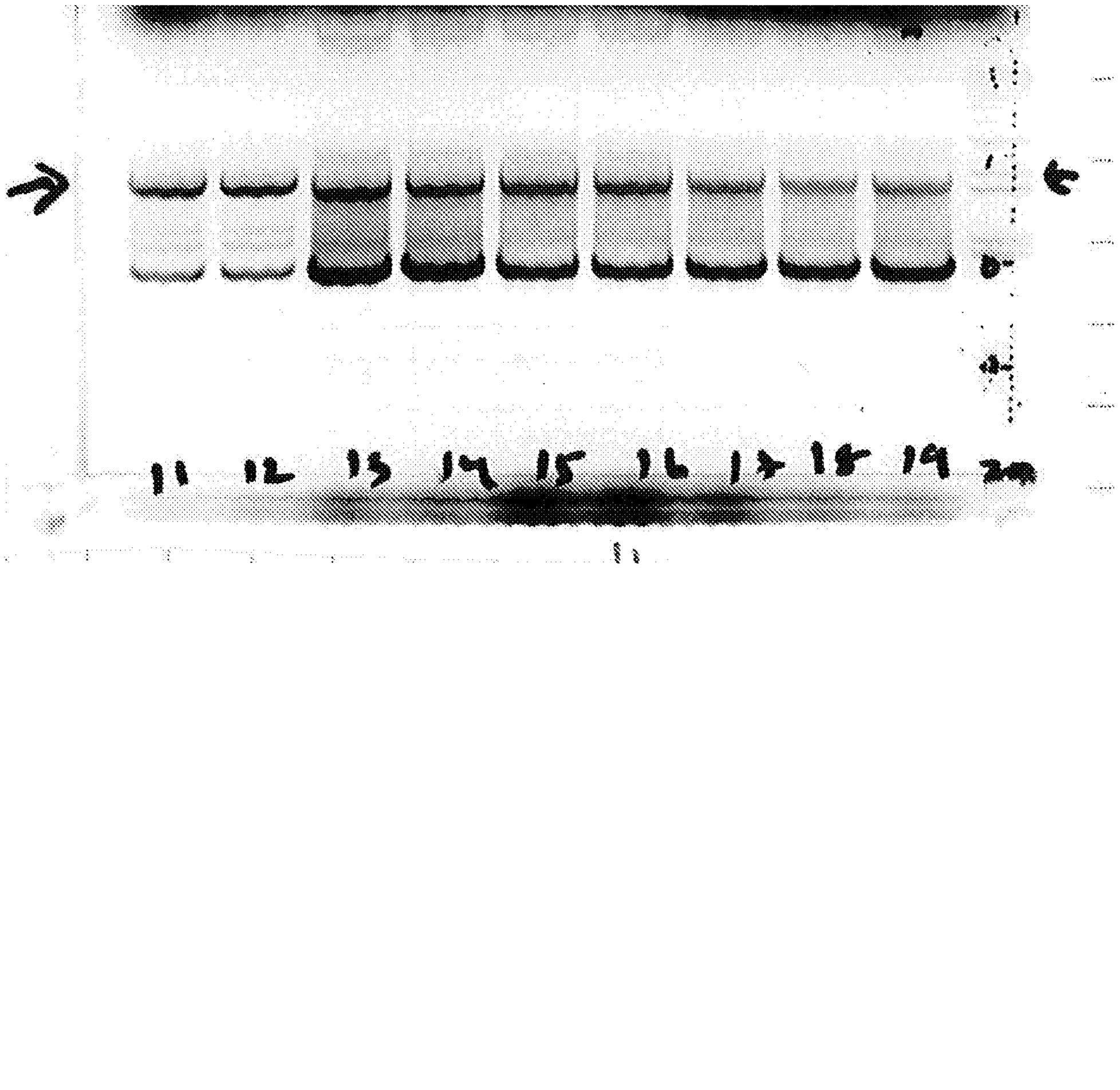


FIG. 2

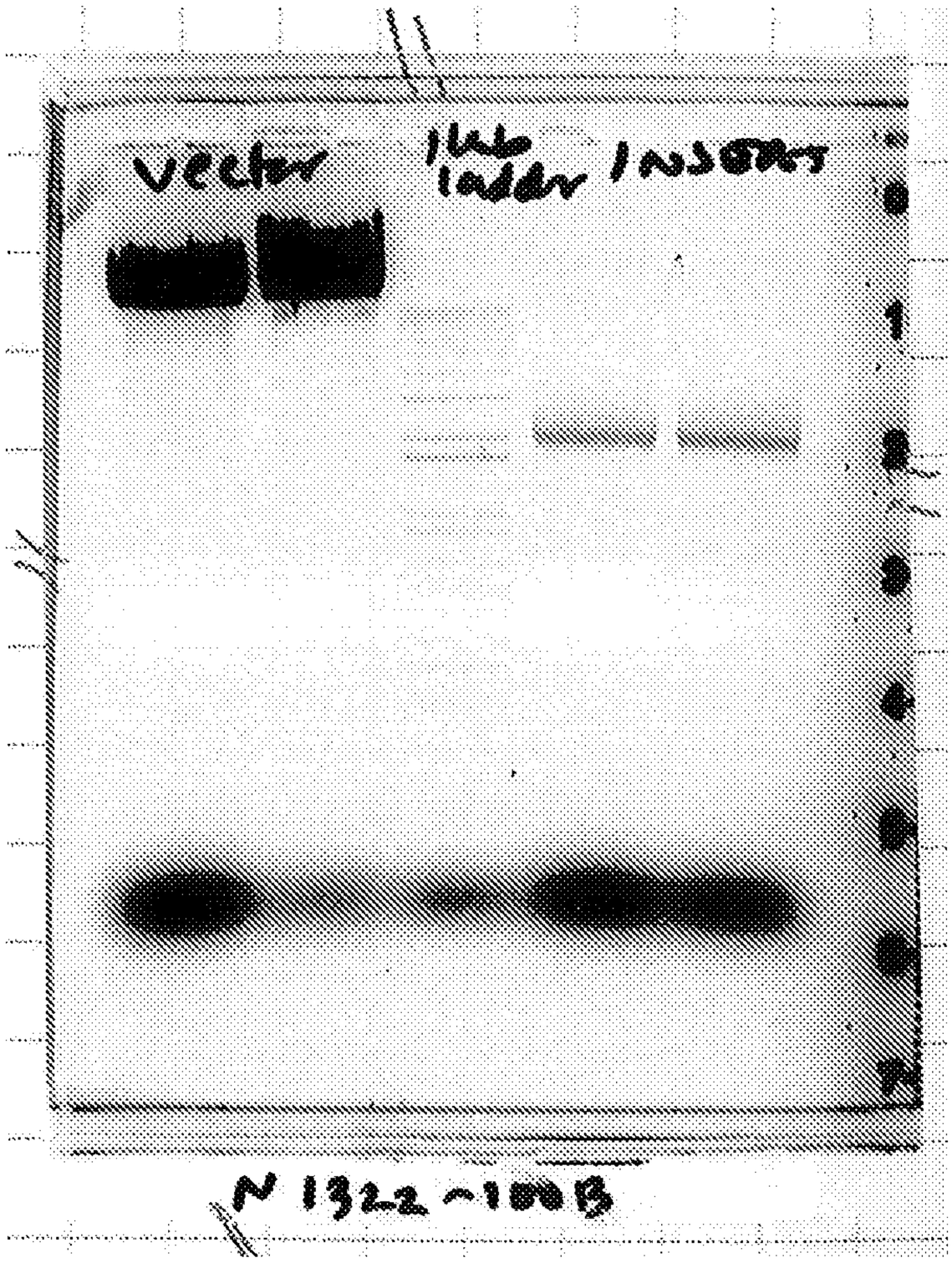


FIG. 3

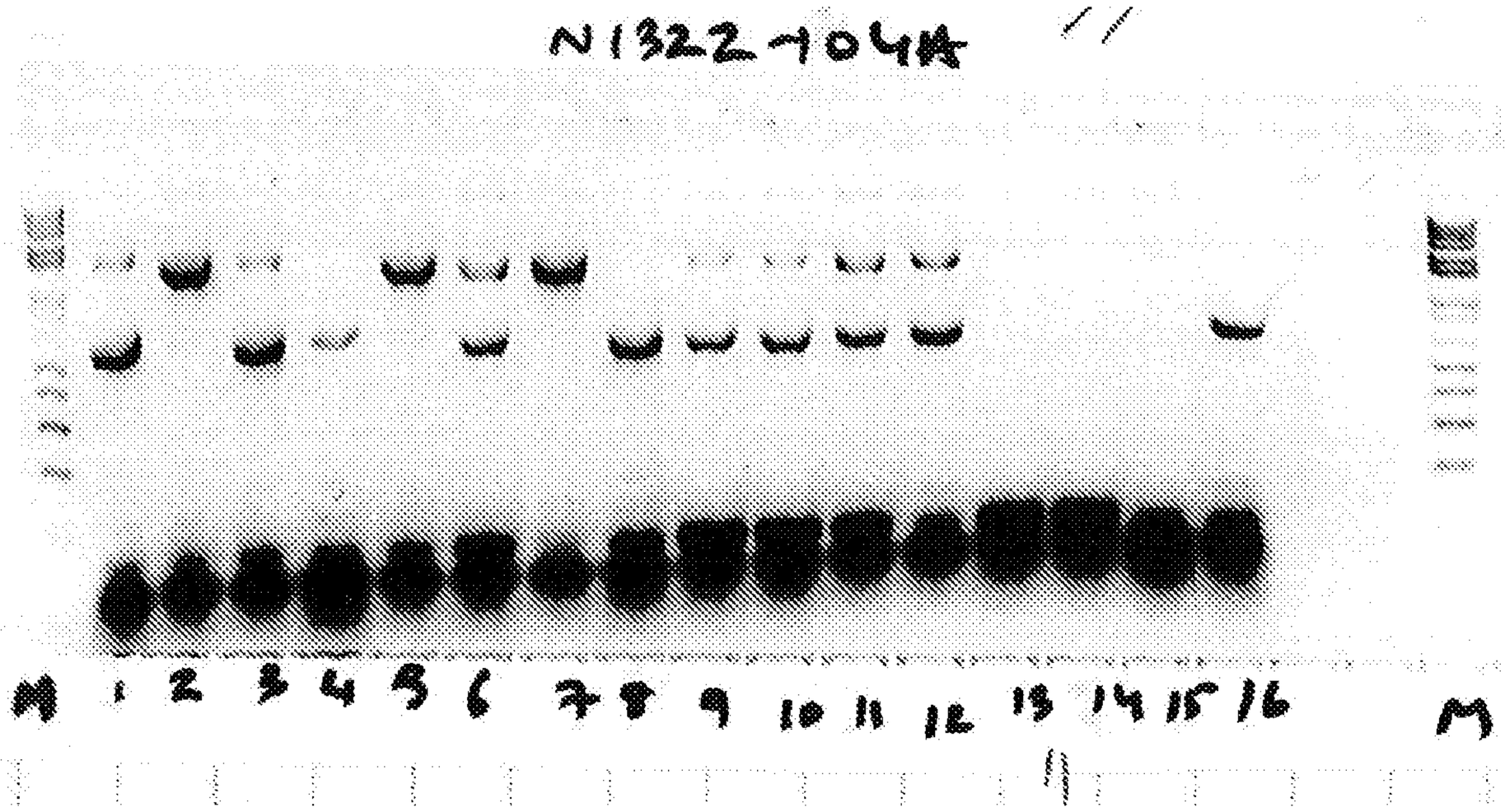


FIG. 4

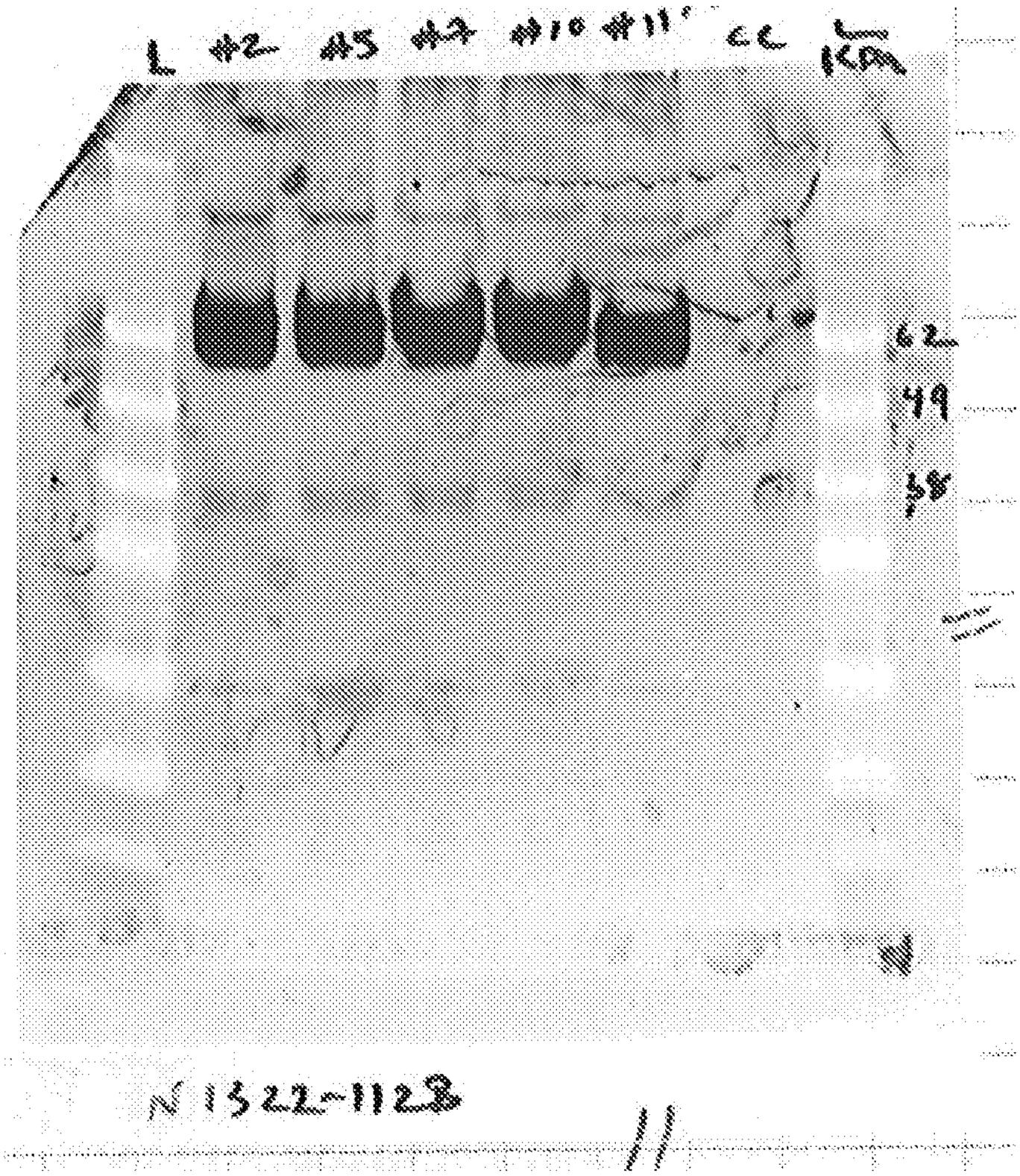
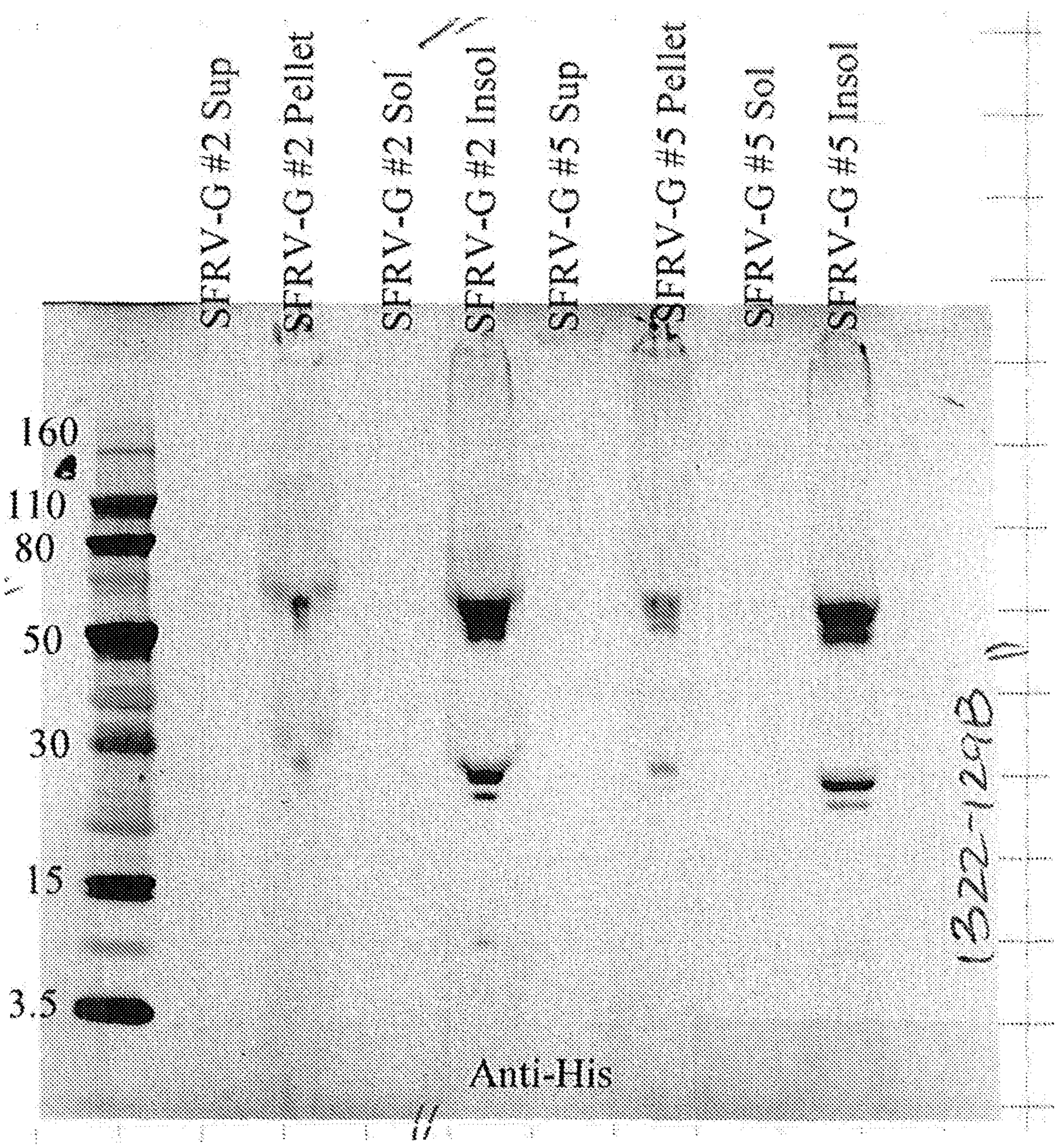
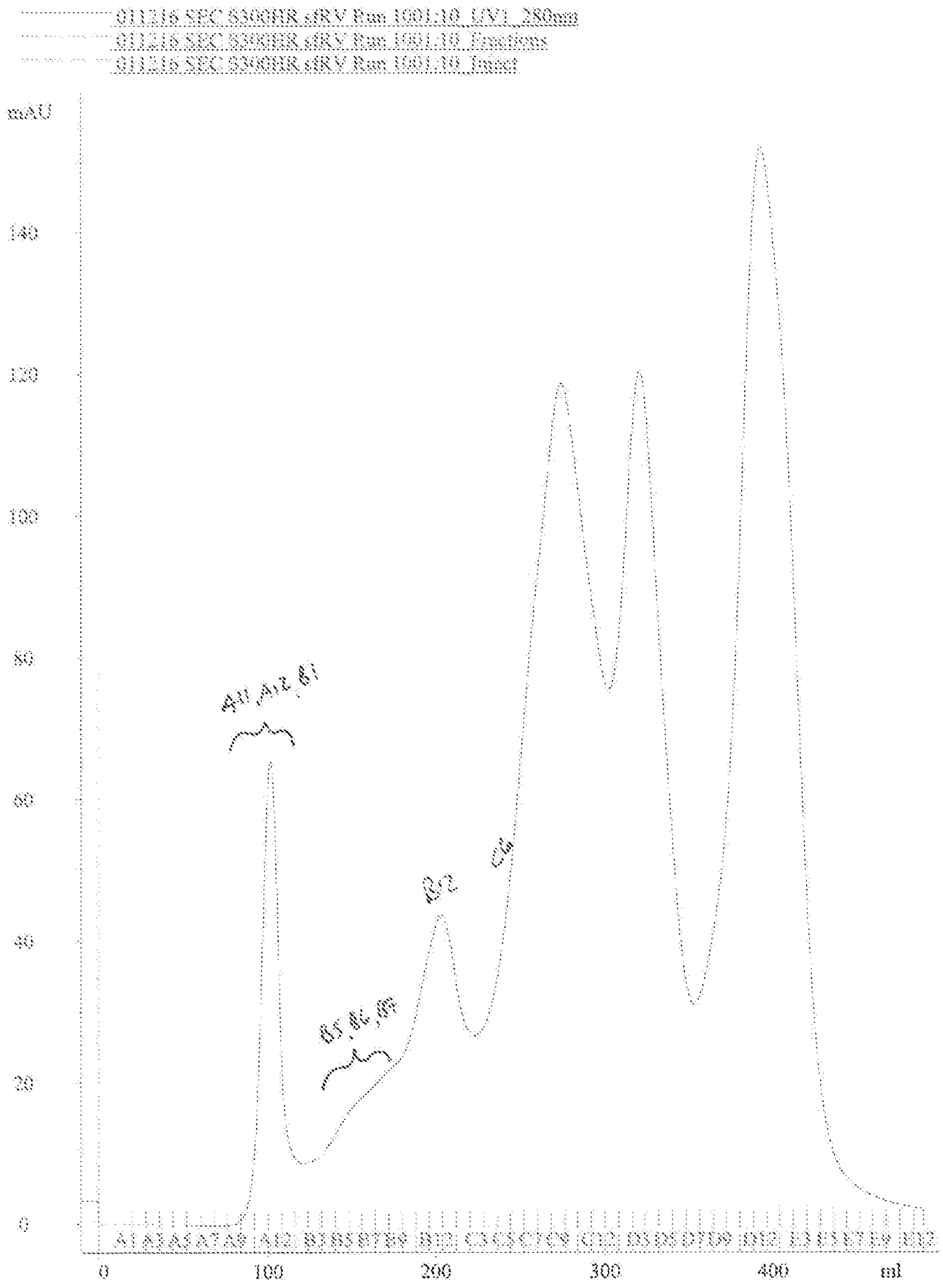


FIG. 5


SFRV-G #2 Sup
SFRV-G #2 Pellet
SFRV-G #2 Sol
SFRV-G #2 Insol
SFRV-G #5 Sup
SFRV-G #5 Pellet
SFRV-G #5 Sol
SFRV-G #5 Insol
160
110
80
50
30
15
3.5
Anti-His
22-129B

6/8

FIG. 6


011216 SEC S300HR cRV Run 1001-10_UV1_280nm
011216 SEC S300HR cRV Run 1001-10_Fractions
011216 SEC S300HR cRV Run 1001-10_Inject
mAU
140
120
100
80
60
40
20
0
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12
0
100
200
300
400
ml

CA 02977405 2017-08-21

FIG. 7

Well #	Fluor	Content	Cq	SQ	
1	FAM	A11	21.41	1.78E+06	Size exclusion fractions
2	FAM	A12	21.95	1.25E+06	
3	FAM	B1	24.45	2.49E+05	
4	FAM	B5	27.31	3.93E+04	
5	FAM	B12	28.67	1.63E+04	
6	FAM	C6	29.98	6.98E+03	
7	FAM	Std	8.49	1.00E+09	Standards
8	FAM	Std	14.92	1.00E+08	
9	FAM	Std	19.54	1.00E+07	
10	FAM	Std	25.98	1.00E+06	
11	FAM	Std	28.26	1.00E+05	
12	FAM	Std	28.40	1.00E+04	
13	FAM	Std	32.54	1.00E+03	
14	FAM	Std	34.53	1.00E+02	
15	FAM	NTC	U	U	Negative control
16	FAM	SfRV +Ctrl	19.22	7.30E+06	Starting material

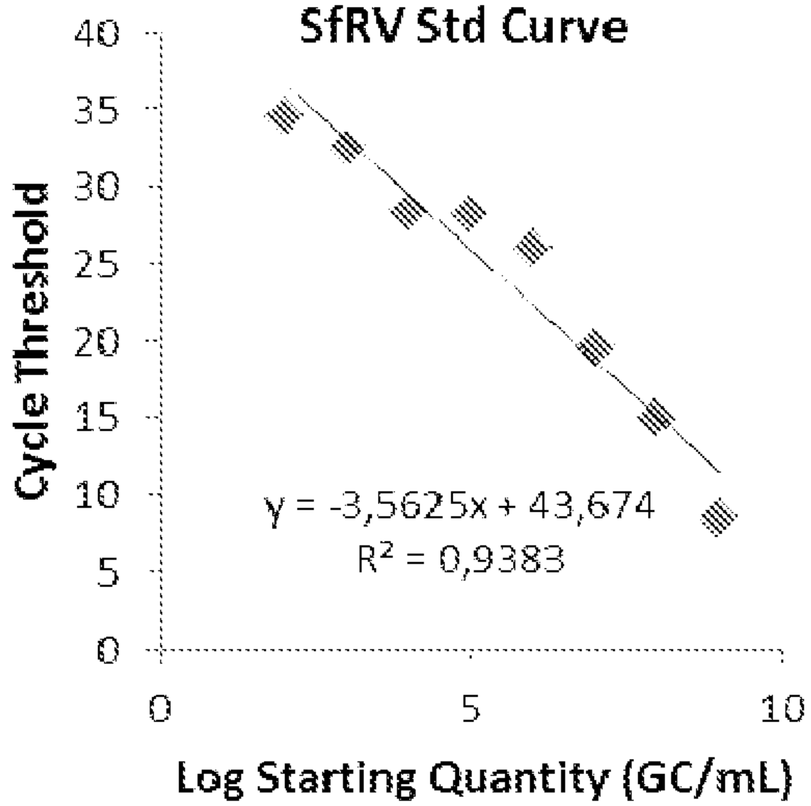
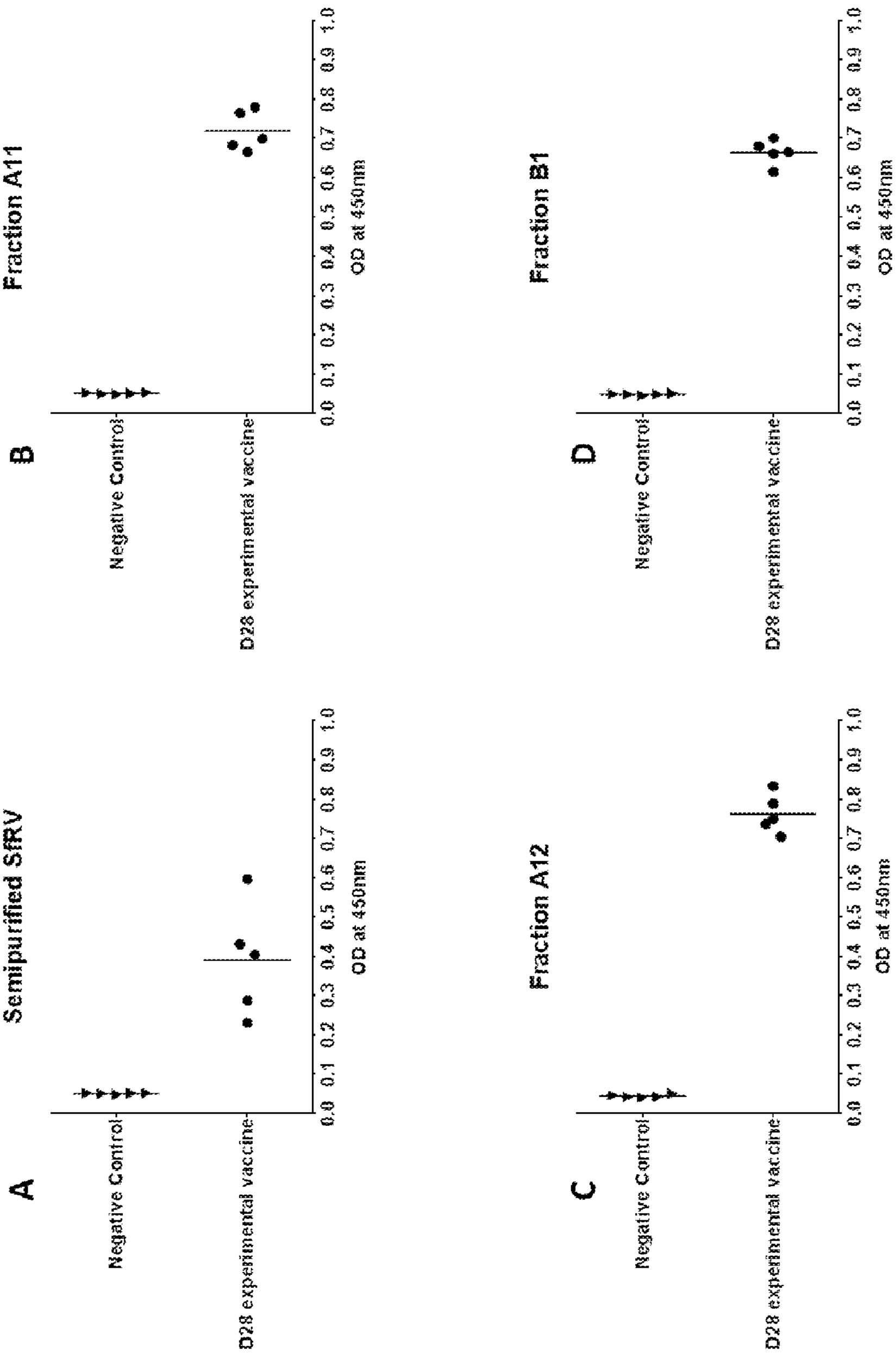


FIG. 8


A
Semipurified SfRV
Negative Control
D28 experimental vaccine
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
OD at 450nm
B
Fraction A11
Negative Control
D28 experimental vaccine
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
OD at 450nm
C
Fraction A12
Negative Control
D28 experimental vaccine
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
OD at 450nm
D
Fraction B1
Negative Control
D28 experimental vaccine
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
OD at 450nm

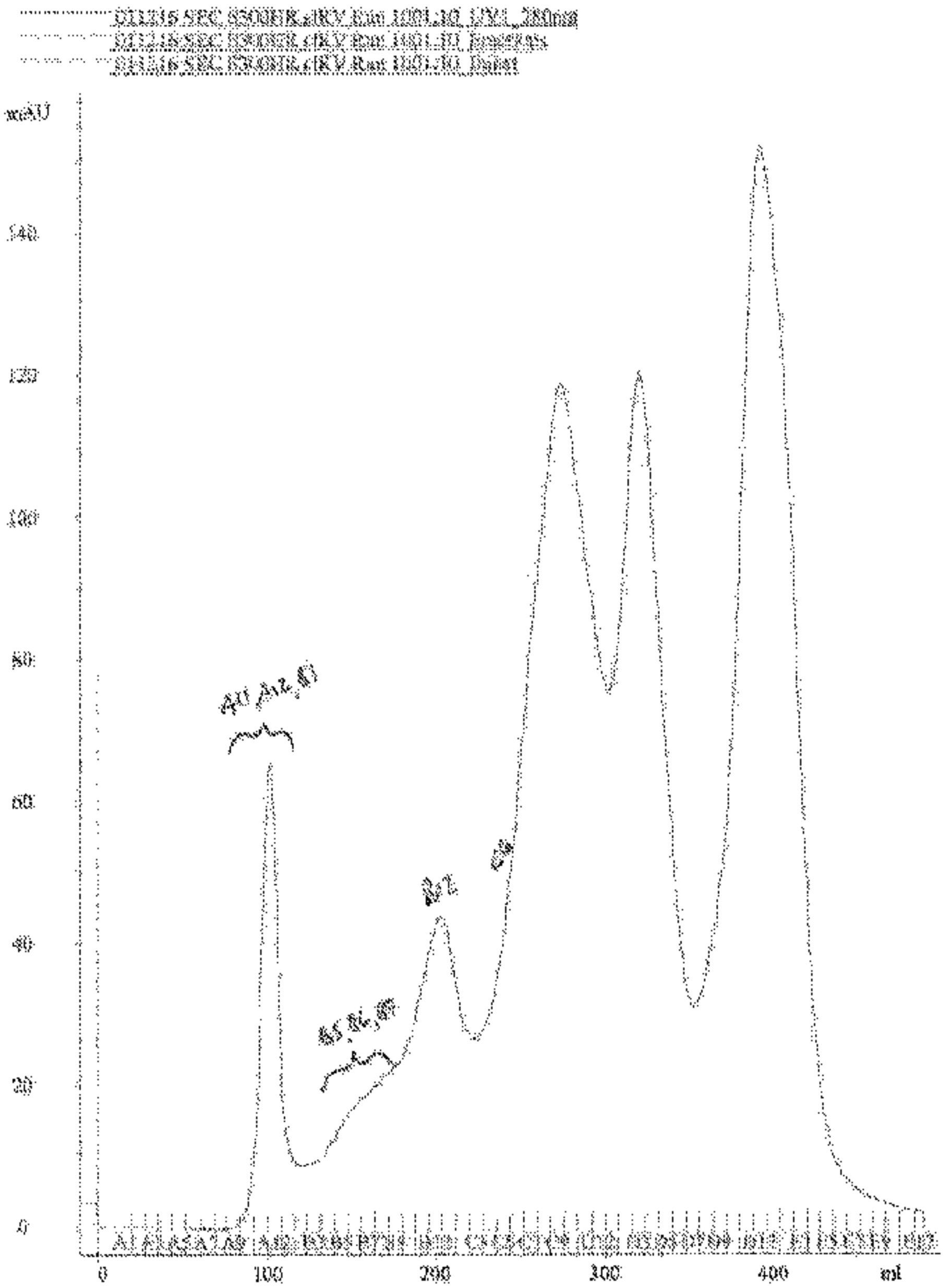



FIG. 6