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(54) Title: TEMPERATURE STABLE VACCINE FORMULATIONS

(57) Abstract: Formulations of vaccine antigen, such as anthrax protective antigen, are provided that are stable after undergoing freeze and thaw conditions. Methods of using the formulations to prepare vaccine are also provided. Vaccines comprising the formulations are useful, for example, to protect against, inhibit or alleviate a disease or infection, such as related to anthrax infection.

TEMPERATURE STABLE VACCINE FORMULATIONS

DESCRIPTION OF THE INVENTION

Government Rights

[0001] This invention was made in part with government support under grant HHSO100201000059C. The government may have certain rights in this invention.

Reference to sequence listing submitted electronically

[0002] The content of the electronically submitted sequence listing in ASCII text file (Name "2479115PC02_sequencelisting.txt"; Size: 12,954 bytes; and Date of Creation: June 25, 2013) filed with the application is incorporated herein by reference in its entirety.

Field of the Invention

[0003] The present invention relates to temperature stable vaccine formulations containing an antigen adsorbed to an aluminum adjuvant and methods of preparing such formulations. The invention includes lyophilized and frozen vaccine formulations. The invention includes temperature stable vaccines, methods of making temperature stable vaccines and methods of use.

Background of the Invention

[0004] Anthrax is a well-known infectious disease caused by a Gram-positive bacterium, *Bacillus anthracis* (*B. anthracis*). Among the three types of anthrax infection (cutaneous, gastrointestinal, and inhalation), cutaneous anthrax is the most common and is relatively easily treatable with various antibiotics. The other two types of anthrax infections are rare, but usually fatal even with aggressive anti-microbial therapy.

[0005] The major virulence factor, anthrax toxin, is composed of three proteins: protective antigen (PA, 83 kilo Dalton, kDa), edema factor (EF, 89 kDa), and lethal factor (LF, 90 kDa). The toxin components act in the binary combinations of PA+EF (edema toxin), and PA+LF (lethal toxin). PA is a cell receptor-binding protein and delivers the other two proteins (EF and LF) into the cytosol of infected cells.

[0006] The most effective known method for preventing anthrax is vaccination. The current and only FDA-approved anthrax vaccine in the United States (produced by Emergent

BioSolutions Inc. under the trademark BioThrax® (Anthrax Vaccine Adsorbed)) is produced from a sterile cell-free filtrate from an avirulent *B. anthracis* V770-NP1-R strain. The licensed anthrax vaccine is also called Anthrax Vaccine Adsorbed (or AVA). The vaccine primarily consists of PA, and aluminum hydroxide is used as an adjuvant. The vaccine was developed during the 1950s and 1960s and is licensed by the FDA to Emergent BioSolutions Inc. The vaccine shows less than 0.06% systemic reactions. The ability of the vaccine to elicit an immune response in humans is well-documented. The AVA vaccine is currently licensed for five doses over 18 months followed by annual boosts.

[0007] Although the AVA vaccine is effective and safe, new immunogenic compositions for preparing a vaccine that protects a subject against a lethal *B. anthracis* infection using recombinant technologies are under development. Because protective antigen (PA) is the common factor required for both the actions of LF and EF, it is often used to prepare vaccines for anthrax. Examples of PA vaccines in development include those disclosed in US patents 6,316,006 and 6,387,665 and patent applications US 2010/0183675, US2011/0229507 and WO2010/053610.

[0008] Vaccines such as an AVA and PA typically contain at least one adjuvant to enhance a subject's immune response. Aluminum salt adjuvants, frequently referred to as alum, are currently the most widely used adjuvant for use in humans. Alum is usually aluminum hydroxide (also marketed as ALHYDROGEL® (aluminum hydroxide) or aluminum phosphate). AVA and the "next generation" Anthrax vaccines (such as recombinant PA) are formulated with ALHYDROGEL which binds the antigen.

[0009] Currently, vaccines containing alum require a cold chain. Cold chains have been established globally to keep vaccines at 2-8° C during storage and distribution. Maintaining cold chains is expensive and difficult. In the event of a cold chain failure, vaccines can be exposed to higher or lower temperatures than intended. It is generally recommended that vaccines that contain alum be discarded if they undergo freeze/thaw processing during shipping and storage. Failure of a cold chain can occur in both industrialized and developing nations, and there are many reasons for cold chain failure, for instance, equipment failure, lack of resources or poor compliance. In many developing countries such as Indonesia, freezing temperatures were recorded in 75% of baseline shipments and freezing of freeze-sensitive is widespread. See *Hepatitis B vaccine freezing in the Indonesian cold chain: evidence and solutions. Bulletin of the World Health Organization 2004; 82:99-105.*

[0010] A vaccine that is dependent on a cold chain may also take longer to distribute to those in need in a timely manner. In the event of a bioterrorist event or other public health emergency, the ability to rapidly deliver vaccines and other medical countermeasures is critical. Eliminating dependence on the cold chain for distribution would lead to more prompt and efficient delivery of medical countermeasures in a variety of climates.

[0011] In order to avoid or minimalize cold chain requirements, many licensed vaccines are formulated as a dry powder composition that can be reconstituted immediately prior to administration. To date, all dry powder vaccines licensed for use in the US are produced through a lyophilization process. Lyophilization, also referred to as freeze drying, is a process that improves the long term stability of a vaccine. The process involves freezing the liquid vaccine formulation and subliming the frozen formulation under vacuum. Other technologies such as spray drying and foam drying have been developed with the aim of producing a stable, dry powder vaccine. These newer technologies produce dry powder vaccine material without the need for freezing and can be used with an alum containing vaccine. See, for instance, *Chen et al., 2010, Vaccine 28:5093-5099.* However, these newer technologies are still in their infancy and have yet to be used in the production of a licensed vaccine in the United States.

[0012] Freezing of vaccine compositions containing alum (either as part of the lyophilization process or to produce a frozen vaccine) generally induces aggregation of the aluminum particles and causes degradation of the antigen adsorbed onto the alum adjuvant resulting in potency loss. In addition, freezing causes reduction of the height of the settled aluminum gel (commonly referred to as gel collapse). See, for instance, "The effect of freezing on the appearance, potency and toxicity of adsorbed and unadsorbed DPT vaccines," 1980, *WHO Weekly Epidemiological Record 55:385-92;* "Temperature Sensitivity of Vaccines," Aug 2006, *WHO publication WHO/IVB/06.10;* Diminsky et al., 1999, *Vaccine 18(1-2):3-17;* Maa et al., 2003, *J Pharm Sci 92(2):319-332.*

[0013] Accordingly, there is a need to produce a vaccine that contains alum that can withstand freezing. Such a vaccine may be subjected to freezing as part of the manufacturing process (*e.g.*, a lyophilized or frozen vaccine), shipping process or during storage. The present invention discloses novel formulations for the production of temperature stable vaccines containing alum.

SUMMARY OF THE INVENTION

[0014] The present invention provides vaccine formulations that contain alum and are capable of being frozen with little to no reduction of potency. In one embodiment, the frozen vaccine composition exhibits little to no alum gel collapse as a result of freezing.

[0015] In one embodiment, the vaccine or composition comprises at least 20% sugar which acts as a stabilizer. In one embodiment, the vaccine or composition comprises greater than 15%, greater than 20%, greater than 25%, or greater than 30% sugar. In some embodiments, the amount of sugar can be reduced without compromising potency if additional stabilizing agents such as amino acids and/or surfactants are added. For frozen and lyophilized vaccine formulations, potency can also be improved by increasing the freezing rate and by freezing suspended particles (as opposed to settled particles).

[0016] The invention includes frozen vaccine compositions, lyophilized vaccine compositions (which undergo freezing as part of the lyophilization process) and other vaccine formulations that are not susceptible to freeze/thaw conditions during shipping and storage.

[0017] Some embodiments of the invention include a composition for preparation of a lyophilized vaccine comprising at least one antigen adsorbed to an aluminum adjuvant and at least 20% (w/v) non-reducing sugar. Another embodiment includes a temperature stable liquid vaccine composition comprising at least one antigen adsorbed to an aluminum adjuvant and at least 20% (w/v) sugar. A further embodiment includes a composition comprising, prior to lyophilization, at least one antigen absorbed to an aluminum adjuvant and at least 20% (w/v) non-reducing sugar, wherein after reconstitution the non-reducing sugar is at least 6% (w/v). Compositions of the invention may further comprise a surfactant. In some embodiments composition of the invention comprises at least one antigen adsorbed to an aluminum adjuvant, a surfactant and at least 15% (w/v) sugar can be used for preparation of a lyophilized vaccine.

[0018] The invention also includes temperature stable liquid vaccine compositions comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant and at least 15% (w/v) sugar. In some embodiments, a composition further comprises an amino acid. Also included are stable liquid vaccine compositions comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant, an amino acid and at least 10% (w/v) sugar.

[0019] The invention further provides compositions for preparation of a lyophilized vaccine comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant, an amino acid and at least 10% (w/v) sugar.

[0020] The invention can be applied to numerous vaccines that contain an antigen adsorbed to an aluminum adjuvant. In one embodiment the vaccine is an anthrax vaccine such as rPA or Anthrax Vaccine Adsorbed.

[0021] The source of the protective antigen may vary. Thus, in some embodiments, the *B. anthracis* protective antigen protein is produced from an asporogenic *B. anthracis* bacterium. In some embodiments, the asporogenic *B. anthracis* bacterium is a ΔSterne-1(pPA102) CR4 strain of bacteria. In some embodiments, PA protein is expressed in other organisms such as E. coli.

[0022] In some embodiments, compositions of the invention may further comprise adjuvants (*e.g.*, in addition to aluminum).

[0023] The present invention includes methods of preventing and treating an anthrax infection comprising administering to a subject a pharmaceutically effective amount of one of the vaccines of the invention. In another embodiment, the invention includes methods of inducing an immune response in a subject comprising administering to the subject a vaccine of the invention.

[0024] The present invention provides method for lyophilizing a vaccine comprising (i) freezing a composition of the invention and (ii) subjecting the frozen composition to sublimation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1. A photograph of an rPA vaccine without a sugar stabilizer at 2° - 8° C compared to the same composition frozen at -80 ° C and thawed.

[0026] Figure 2. A photograph of an rPA vaccine with 20% trehalose and 2% arginine at 2° - 8° C compared to the same composition frozen at -80 ° C and thawed.

[0027] Figure 3. Photographs of an rPA vaccine with and without sucrose at 2° - 8° C and at -80° C followed by thawing.

[0028] Figure 4. A graph showing the GeoMean NF50 of a no sugar rPA formulation before and after freezing and photographs showing the collapse of the alum gel.

[0029] Figure 5. A graph showing the GeoMean NF50 response of lyophilized samples with and without sugar that were not frozen.

[0030] Figure 6. A graph showing the GeoMean NF50 before and after freeze/thaw for an rPA composition containing 20% trehalose.

[0031] Figure 7. A graph showing GeoMean NF50 for all lyophilized samples compared to a liquid control.

[0032] Figures 8A-B. A graph showing NF50 over time for lyophilized rPA at (A) 5°C and 50°C compared to AVA shown in linear NF50 scale and (B) 5°C and 50°C compared to AVA shown in log NF50 scale, each vaccine was at a 1:4 dilution.

[0033] Figures 9A-B. A graph showing NF50 over time for lyophilized rPA at (A) 5°C and 50°C compared to AVA shown in linear NF50 scale and (B) 5°C and 50°C compared to AVA shown in log NF50 scale, each vaccine was at a 1:16 dilution.

[0034] Figures 10A-B. A graph showing NF50 over time for lyophilized rPA at (A) 5°C and 50°C compared to AVA shown in linear NF50 scale and (B) 5°C and 50°C compared to AVA shown in log NF50 scale, each vaccine was at a 1:64 dilution.

[0035] Figures 11A-B. A graph showing the comparison of NF50 at (A) day 35 and (B) day 42 for lyophilized rPA at 5°C and 50°C and AVA, each vaccine was at a 1:4 dilution.

[0036] Figures 12A-B. A graph showing the comparison of NF50 at (A) day 35 and (B) day 42 for lyophilized rPA at 5°C and 50°C and AVA, each vaccine was at a 1:16 dilution.

[0037] Figures 13A-B. A graph showing the comparison of NF50 at day 35 (A) day 35 and (B) day 42 for lyophilized rPA at 5°C and 50°C and AVA, each vaccine was at a 1:64 dilution.

[0038] Figure 14. A graph showing a comparison of the %MLA (microphage lysis assay) value for rPA liquid vaccine (Liq rPA F1) stored at one month versus lyophilized vaccines (LyoA, LyoB, and LyoC) stored at four months as a function of temperature.

[0039] Figures 15A-B. (A) A graph showing the relative drop in SEC %purity over reference control as a function of storage temperature of three rPA lyophilized formulations (LyoA, LyoB, and LyoC) stored for four months compared to liquid rPA stored for one month. (B) Shows a typical size exclusion chromatography (SEC-HPLC) chromatograph of rPA BDS reference standard.

[0040] Figures 16A-B. (A) A graph showing the relative drop in %AEX purity as a function of storage temperature of three rPA lyophilized formulations (LyoA, LyoB, and LyoC) stored for four months compared to liquid rPA stored for one month. (B) Shows an

anion exchange chromatography (AEX-HPLC) chromatographs of rPA BDS reference standard.

[0041] Figures 17A-D. Graphs showing the comparison of NF50 at dose level 0.25 (Panel A), 0.125 (Panel B), 0.0625 (Panel C) and 0.03125 (Panel D) for LyoA stored at 5, 25, and 40 °C for one month.

[0042] Figures 18A-D. Graphs showing the comparison of NF50 at dose level 0.25 (Panel A), 0.125 (Panel B), 0.0625 (Panel C) and 0.03125 (Panel D) for LyoB stored at 5, 25, and 40 °C for one month.

[0043] Figures 19A-D. Graphs showing the comparison of NF50 at dose level 0.25 (Panel A), 0.125 (Panel B), 0.0625 (Panel C) and 0.03125 (Panel D) for LyoC stored at 5, 25, and 40 °C for one month.

[0044] Figures 20A-D. Graphs showing the comparison of NF50 at dose level 0.25 (Panel A), 0.125 (Panel B), 0.0625 (Panel C) and 0.03125 (Panel D) for liquid rPA stored at 5, 25, and 40 °C for one month.

[0045] Figure 21. A graph showing NF50 values and the standard deviation of mean for 12 formulations as described in Example 8.

DETAILED DESCRIPTION

[0046] For many years it has been believed that alum containing vaccines cannot be frozen. Accordingly, alum containing vaccines are not frozen or lyophilized (requires freezing), and alum-containing liquid vaccines are typically discarded if a break in the cold chain causes freezing. The inventors of the present invention made the exciting discovery that when a sugar such as trehalose or sucrose makes up about 20% (w/v) or more of an anthrax vaccine composition, the alum in the composition does not collapse as a result of freezing or thawing. Alum collapse is easy to identify and is associated with loss of vaccine potency and particle aggregation.

[0047] The inventors also identified additional stabilizing ingredients and process parameters that help prevent and reduce alum gel collapse. By adding amino acids to a formulation for instance, the amount of sugar required to prevent alum gel collapse can be reduced, *e.g.*, to about 10% (w/v). Process changes that have a positive effect on alum gel height include freezing suspended particles (rather than settled particles) and increasing the freeze rate.

[0048] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited herein, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated documents or portions of documents defines a term that contradicts that term's definition in the application, the definition that appears in this application controls.

[0049] The use of the singular includes the plural unless specifically stated otherwise. The word "a" or "an" means "at least one" unless specifically stated otherwise. The use of "or" means "and/or" unless stated otherwise. The meaning of the phrase "at least one" is equivalent to the meaning of the phrase "one or more." Furthermore, the use of the term "including," as well as other forms, such as "includes" and "included," is not limiting. Also, terms such as "element" or "component" encompass both elements or components comprising one unit and elements or components comprising more than one unit unless specifically stated otherwise. The word "about" means within about 1 unit.

[0050] As used herein, Protective Antigen (PA) or recombinant Protective Antigen (rPA) is the component of anthrax toxin (approx 83 kDa) that contains the receptor-binding and translocation domains. One example of a full length PA amino acid sequence is:

EVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVVTSSTTGDLSIPSSEL
ENIPSENQYFQSAIWSGFIKVKKSDEYTFATSADNHVTMWVDDQEVINKA
SNSNKIRLEKGRLYQIKIQYQRENPTEKGLDFKLYWTDSQNKKEVISSDN
LQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYTVDVKNKR
TFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNVSPE
ARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSQTRTISKNTSTSRTHT
SEVHGNAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETM
GLNTADTARLNANIRYVNTGTAPIYNVLPTTSLVLGKNQTLATIKAKENQ
LSQILAPNNYYPSKNLAPIALNAQDDFSSTPITMNYNQFLELEKTKQLRL
DTDQVYGNIATYNFENGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNL
VERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPNGNLQYQGKDITEF
DFNFDQQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKRFHYDR
NNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTE
GLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVYA
VTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG

(SEQ ID NO: 1).

[0051] SEQ ID NO: 1 is the amino acid sequence of rPA102 which is expressed from plasmid pPA102. During secretion of rPA102 from *B. anthracis* ΔSterne-1(pPA102)CR4 into the extracellular space, the first 29 amino acids (the signal peptide) are removed yielding the mature rPA protein of 735 amino acids (82,674 Da). The mature rPA sequence is underlined (SEQ ID NO: 2).

[0052] The rPA102 amino acid sequence is but one example of one particular anthrax protein within the scope of the invention. Additional amino acid sequences of PA proteins, including native proteins, from various strains of anthrax are known in the art and include, for example, GenBank Accession Nos: NP_652920.1, ZP_02937261.1, ZP_02900013.1, ZP_02880951.1 which are incorporated by reference. Various fragments, mutations, and modifications in PA to reduce its toxicity or to improve its expression characteristics are also known, such as those described elsewhere in the specification, as are various fusion proteins. Those fragments, mutants, and fusion proteins are included in the term "PA" unless the context or text clearly indicates that those forms are excluded. Where indicated, PA fragments, mutants, and fusion proteins (whether with full length PA or a PA fragment) are those that elicit an antisera that is active in the toxin neutralization assay (TNA).

[0053] As used herein, "temperature stable," "stable" or "stability" refers to the stability of the alum gel and potency of a vaccine after a freeze/thaw cycle. A stable vaccine as used herein is a vaccine that exhibits no or little decrease in activity and/or potency and/or alum gel collapse and/or particle aggregation after a freeze/thaw cycle as compared to a comparable liquid vaccine that is kept between 2°- 8° C. Stability can be measured using any one or more of the assays described herein, including the working examples, as well as assays known in the art that are used to measure activity, potency and/or peptide degradation.

[0054] In certain embodiments, the immunogenicity of an antigen or vaccine, e.g., protective antigen, can be measured by calculating 50% neutralization factor (NF50). The geometric mean of the NF50 (GeoMean or <NF50>gm) for vaccine formulation can be calculated based on the NF50 values from a given number of data points. In certain embodiments, the NF50 and/or GeoMean is determined by using serum samples from a standard Toxin Neutralization Assay (TNA) (Hering et al., Biologicals 32 (2004) 17-27; Omland et al., Clinical and Vaccine Immunology (2008) 946-953; and Li et al., Journal of Immunological Methods (2008) 333:89-106), e.g., serum from immunized mice or rabbits. The dilution of serum resulting in 50% neutralization of toxin is the "ED50". The

neutralization capacity of each test serum in relation to that of a reference serum (50% neutralization factor, or NF50, also known as the neutralization ratio) is calculated from the quotient of the ED50 of the reference serum and the ED50 of the test serum, i.e., the neutralization factor, NF50 is calculated as follows:

$$NF50 = \frac{ED50_{sample}}{ED50_{reference}}$$

[0055] In certain embodiments, a T-test or one-way ANOVA can be used to compare the geometric mean of NF50 from different formulation at the 95% confidence level. In one embodiment, if the p value of the GeoMean NF50 is larger than 0.05, there is no significant difference in NF50 among the formulations. In another embodiment, if the p value is less than 0.05, the geometric mean NF50 among the formulations is significantly different from each other.

[0056] Neutralization factor (NF50) calculations from mouse potency assay experiments show that the NF50 values and thus potency correlate with alum gel collapse. Accordingly, stability can be assessed by observing and measuring the alum gel of a vaccine that has been frozen overnight at -80° C and then allowed to thaw at room temperature. A stable vaccine will exhibit little to no alum gel collapse as compared to a control vaccine (same composition but stored at 2° - 8° C). Alum gel height can be measured and a % difference between the frozen/ thawed sample and 2° - 8° C control can be determined. In one embodiment, a difference of about less than 1%, 2%, 3%, 5%, 8%, 10% or 12% indicates a stable vaccine.

[0057] Stability can also be assessed by assaying the composition after freezing for intact protein (e.g., rPA intact with alum) or, conversely, desorbed protein (e.g., rPA desorbed from alum. For instance, stability can be determined by assaying and characterizing free rPA102 (release) by ELISA; protein structure by, for instance, differential scanning calorimetry and intrinsic fluorescence; desorbed free protein by A_{280}; purity and backbone degradation by SDS-PAGE, SEC and or RP-HPLC; charge variation by IEX or isoelectric focusing; and biochemical activity by microphage lysis assay (MLA).

[0058] In one embodiment, a temperature stable vaccine is a vaccine that after being exposed to freeze/thaw conditions (e.g., frozen vaccine or lyophilized vaccine), exhibits potency that is the same or at least about 98%, at least about 95%, at least about 93%, at least about 90%, at least about 88% or at least about 85% the same as a comparable liquid vaccine stored at about 2° - 8° C. In one embodiment, an anthrax mouse potency assay is used to determine whether a frozen or lyophilized vaccine is potent.

[0059] In some embodiments, a composition retains at least 80%, at least 90% or at least 95% immunogenicity after storage in lyophilized form for at least 1 month at 40 °C.

[0060] The vaccines of the invention are temperature stable vaccines. The temperatures over which a formulation of the invention is stable are generally below about 30° C, but may be above 30° C, 35° C, 40° C, 45° C, or 50° C. In some embodiments, the formulation's stability is in reference to a temperature below about 25° C, about 20° C, about 15° C, about 10° C, about 8° C, about 5° C, about 4° C, or about 2° C. Thus, in some embodiments, the temperature is in the range of about 25° C to about -10° C, about 20° C to about -10° C, about 15° C to about -10° C, about 10° C to about -10° C, about 8° C to about -10° C, about 5° C to about -10° C, about 15° C to about -5° C, about 10° C to about -5° C, about 8° C to about -5° C, and about 5° C to about -5° C.

[0061] The Examples section describes various methods for determining stability. In some embodiments, a vaccine of the invention shows no statistically significant decrease in stability after freeze thaw as compared to the same sample but fresh and/or stored 5 °C. In some embodiments, a vaccine of the invention shows no statistically significant decrease in stability, immunogenicity, potency or any combination thereof after storage at -80° C, -20° C, 25° C, 40° C and/or 50° C for 1, 2, 3, 4, 5, 6, 9 12, 18, 24, 30, 36, 42, 48, 54 or 60 months as compared to storage at 5° C for the same time period.

[0062] In some embodiments, the stability of a composition is measured by microphage lysis assay (MLA), size exclusion chromatography (SEC-HPLC) and/or anion exchange chromatography (AEX-HPLC).

[0063] In some embodiments, a composition retains at least 80%, at least 90% or at least 95% purity after storage in lyophilized form for at least 4 months at 50 °C.

[0064] The vaccine compositions of the invention contain an antigen which is adsorbed to an aluminium adjuvant (alum) and an amount of sugar necessary to stabilize the formulation. For instance, the vaccine formulations disclosed herein exhibit little to no reduction in potency after exposure to freeze/thaw conditions when compared to a similar liquid vaccine that has been maintained at between 2°- 8°C and /or exhibit little to no collapse of alum gel.

[0065] The aluminium adjuvant (alum) can be, for instance, aluminium hydroxide, aluminium phosphate or aluminium sulphate. In one embodiment, the adjuvant is aluminium hydroxide (*e.g.*, ALHYDROGEL). The amount of aluminium can vary quite a bit with apparently no effect on the stability of the alum gel (in other words, increasing the amount of alum in the composition does not appear to increase the likelihood that the alum gel will

collapse). In one embodiment of the invention, the vaccine composition comprises about 1-10 mg/ml aluminium hydroxide. In another embodiment, the composition comprises about 1.5 to 5 mg/ml aluminium hydroxide. In another embodiment, the vaccine composition comprises about 1.5, 2, 2.5, 3, 3.5, 4, 4.5 or 5 mg/ml aluminium hydroxide.

[0066] It is believed that the stable vaccine compositions of the invention can be used to stabilize any antigen that is formulated with alum. For instance, the antigen can be a *B. anthracis* recombinant Protective Antigen (rPA) or a cell-free filtrate from an avirulent *B. anthracis* strain such as V770-NP1-R (*e.g.*, anthrax vaccine adsorbed).

[0067] Methods of expressing *B. anthracis* proteins, including PA (as well as fragments, mutants, and fusion proteins) are described, for example in U.S. Patent No. 7,201,912, to Park and Giri, U.S. Patent No. 6,387,665 to Ivins *et al.*, U.S. Patent No. 6,316,006 to Worsham *et al.*, and U.S. Patent No. 7,261,900 to Leppla *et al.*, each of which is incorporated by reference in its entirety. For example, as described in U.S. Patent No. 7,201,912, pBP103 is an expression vector for full-length, wild-type rPA. The PA sequence from pBP103 is identical to that of wild-type PA.

[0068] Some embodiments of the invention include formulations comprising PA expressed in *B. anthracis*, including expression in either sporulating or non-sporulating strains of *B. anthracis* or both. For instance, the PA can be derived from non-sporulating *B. anthracis* strain ΔSterne-1 (pPA102)CR4 (*i.e.*, rPA102). See, for instance, U.S. Patent No. 6,316,006 and U.S. Patent No. 6,387,665, both to Ivins *et al.*, each of which is herein incorporated by reference in its entirety. Some compositions of the invention comprise a PA from the avirulent B. anthracis strain V770-NP1-R

[0069] The formulations of the invention may also include *B. anthracis* PA expressed by a heterologous organism. For instance, the invention includes PA expressed in *E. coli*.

[0070] In addition, various PA fragments, mutants, and fusion proteins have also been described and can be used in the current formulations. For example, PA may be modified to lack a functional binding site, thereby preventing PA from binding to either Anthrax Toxin Receptor (ATR) (see Bradley, K.A., Nature (2001) 414:225-229) to which native PA binds, or to native LF. By way of example, a modification made within or near to amino acid residues 315-735 or within or near to residues 596-735 of Domain 4 may render PA incapable of binding to ATR. Alternatively (or in addition), the PA furin cleavage site "RKKR" (SEQ ID NO: 3), which in most full length PA sequences is found at or around residues 163-168, may be inactivated by deletion, insertion, or substitution within or near to

the furin cleavage site. For example, all of the furin cleavage site residues of native PA may be deleted. Other mutant PAs include those in which the dipeptide Phe-Phe has been modified to render the PA resistant to chymotrypsin. A PA fragment or PA fusion protein may also be a PA mutant.

[0071] Specific examples of PA fragments include those in U.S. Patent No. 7,201,912, for example, PA64 expressed by pBP111, PA47 expressed by pBP113, PA27 expressed by pBP115. Some of those fragments may also include mutations to, for example, eliminate the furin cleavage site RKKR (SEQ ID NO: 3) or the chymotrypsin sensitive site formed by the dipeptide sequence Phe-Phe (FF). In addition, fragments may include one or two additional amino acids at the N-terminus. Examples of fusion proteins involving PA include those in U.S. Patent No. 7,201,912, for example the PA-LF fusion proteins expressed by plasmids pBP107, pBP108, and pBP109. The invention also includes formulations comprising a HIS-tag PA. When a fragment, mutant, or fusion protein is used, however, it is generally desirable that the fragment, mutant, or fusion protein elicit protective immunity to a challenge with, *e.g.*, an LD_{50}, of anthrax spores of the Ames strain in one or more of mice, guinea pigs, or rabbits.

[0072] PA from a recombinant source and/or a non-recombinant source can be used and the stability of such preparations improved by the formulations of the invention.

[0073] In one embodiment, the vaccine composition comprises about 75 to 750 μg/ml, 100 to 500 μg/ml, 100 to 250 μg/ml, 100 to 750 μg/ml or 250 to 750 μg/ml of antigen, *e.g.*, rPA. For instance, the invention includes a vaccine comprising about 150, 200, 250, 300, 350, 400, 450 and 500 μg/ml of antigen, *e.g.*, rPA. In some embodiments, the vaccine comprises approximately 175 μg antigen (*e.g.*, rPA) per 1500 μg aluminum hydroxide. In some embodiments, the vaccine comprises approximately 200 μg/mL antigen (*e.g.*, rPA) and about 0.5 mg/mL aluminum hydroxide. In further embodiments, the vaccine comprises approximately 250 μg antigen (*e.g.*, rPA) per 100 to 250 μg aluminum hydroxide. In some embodiments, an antigen is an Anthrax antigen such as protective antigen. In some embodiments, a protective antigen is at least about 80% identity to the polypeptide of SEQ ID NO: 2. Some compositions of the invention comprise about 150-500 μg/ml protective antigen or about 150, 175, 200, 225, 250, 275, 300, 325, 400, 375, 400, 425, 450, 475 or 500 μg/ml protective antigen.

[0074] In some embodiments a composition of the invention contains about 0.5 to 1.5 mg/ml aluminum hydroxide. In some embodiments, a composition contains about 0.5 mg/ml or about 1.5 mg/ml aluminum hydroxide.

[0075] In some embodiments, an aluminum adjuvant is selected from the group consisting of aluminum hydroxide, aluminum phosphate and aluminum sulfate.

[0076] An anthrax vaccine of the invention, whether it be a vaccine comprising rPA or a cell-free filtrate from an avirulent *B. anthracis* strain, can be administered to a subject pre-exposure or post-exposure to *B. anthracis*. When administered post-exposure, the vaccine may be administered in conjunction with antibiotics.

[0077] In another embodiment, the antigen is a protein (*e.g.*, recombinant) based antigen selected from the group consisting of hepatitis B protective antigens, *Clostridium botulinum* neurotoxin protein, Herpes Simplex Virus antigens, Influenza antigens, Congenital cytomegalovirus antigens, Tuberculosis antigens, HIV antigens, Diphtheria antigens, Tetanus antigens, Pertussis antigens, *Staphylococcus* enterotoxin B (SEB), and *Yersinia pestis* protective antigens and F1-V fusion protein. Antigens can be derived, for instance, from papillomavirus (*e.g.*, HPV), influenza, a herpesvirus, a hepatitis virus (*e.g.*, a hepatitis A virus, a hepatitis B virus, a hepatitis C virus), *Meningococcus A, B and C*, *Haemophilus influenza* type B (HIB), *Helicobacter pylori*, *Vibrio cholerae*, *Streptococcus sp.*, *Staphylococcus sp.*, *Clostridium botulinum*, *Bacillus anthracis* and *Yersinia pestis*.

[0078] The vaccines of the present invention can withstand freezing overnight at -80° C with little to no loss of potency or collapse of alum gel. The invention includes frozen liquid vaccines as well as lyophilized vaccines (also referred to herein as freeze dried vaccines). As disclosed herein, the lyophilization process includes the freezing of a liquid composition. The frozen composition is then subjected to sublimation under freezing. For the lyophilized vaccines, the disclosed vaccine components and amounts refer to the amounts used in the liquid composition that is then subjected to freezing and not necessarily the dried lyophilized cake or reconstituted vaccine. The final lyophilized vaccine cake (a dry composition) may contain different percentages of components due to the drying process.

[0079] The present invention provides method for lyophilizing a vaccine comprising (i) freezing a composition of the invention and (ii) subjecting the frozen composition to sublimation.

[0080] In one embodiment, the vaccine of the invention comprises about 20% or more of a glass forming agent such as sugar. In one embodiment, the glass-forming agent is a

reducing sugar. In one embodiment, the vaccine comprises a non-reducing sugar such as trehalose or sucrose. In one embodiment, the glass forming agent is trehalose or sucrose. If the vaccine is lyophilized, it may be preferable to use no more than about 40% sugar, prior to lyophilization, so that the vaccine forms a cake-like composition. The vaccine may comprise about 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35% or 40% sugar, *e.g.*, prior to lyophilization. In one embodiment, the vaccine composition comprises about 10-40%, 10-35%, 10-30%, 10-25%, 10-20%, 35-40%, 30-40%, 25-40%, 20-40%, 15-40%, 20-30%, 20-25%, 25-30%, 25-35%, 21-40%, 21-35%, 21-30% 21-25% or greater than 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, or 36% (w/v) sugar, *e.g.*, prior to lyophilization. In some embodiments, a composition contains greater than about 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24% and 25% (w/v) sugar, *e.g.*, prior to lyophilization.

[0081] As disclosed herein, the inventors of the present invention have identified that alum vaccine compositions comprising at least about 20% trehalose or sucrose can withstand freeze/thaw conditions. When certain additional stabilizing agents are added (*e.g.*, a surfactant and/or amino acid) and/or the process improvements disclosed herein are incorporated (*e.g.*, increasing freeze rate, freezing of suspended particles), the amount of sugar can be reduced to about 15% (w/v) or even about 10% (w/v) without affecting potency of the vaccine.

[0082] In one embodiment of the invention, a vaccine composition comprising an antigen adsorbed to an aluminium adjuvant and a sugar (*e.g.*, 15% w/v or more) also contains a solubilizing agent such as a surfactant, *e.g.*, prior to lyophilization. In one embodiment, the surfactant is a nonionic detergent such as polysorbate 80 (TWEEN® 80). In one embodiment, the vaccine composition comprises between about 0.001% and about 0.05% surfactant (such as polysorbate 80). In one embodiment, the composition comprises about 0.020%, about 0.025% or about 0.020% to 0.025% (w/v) surfactant (such as polysorbate 80). Other surfactants that can be used include, but are not limited to, polysorbate 20, pluronic L68, polyoxyethylene 9-10 nonyl phenol (Triton™ N-101, octoxylnol 9), Triton™ X-100, and sodium dexoycholte. In one embodiment, the surfactant is removed during the manufacturing process so that no surfactant is present in the final drug product. In one embodiment, a surfactant is present during freezing, *e.g.*, of lyophilization process. In some embodiments, a formulation of the invention does not comprise a surfactant.

[0083] The inventors have found that the percentage of sugar may be reduced to as much as about 10% (w/v) with little to no effect on potency and/or little to no alum gel collapse if amino acids (for instance, alanine, arginine, glycine and proline) are added to the composition, *e.g.*, prior to freezing and/or lyophilization. The amount of amino acid added to the vaccine composition can vary. In one embodiment, the vaccine composition comprises 0.5 to about 15% (w/v) of an amino acid or combination of amino acids. In one embodiment, the vaccine composition comprises about 2-10% (w/v) of an amino acid or combination of amino acids. In one embodiment of the invention, the vaccine comprises about 2% arginine or alanine. In another embodiment of the invention, the vaccine comprises about 10% glycine. Is some embodiments, a vaccine composition comprises about 2-10%, 2-8%, 2-6%, 2-4%, 2-3%, 3-10%, 5-10%, 7-10%, 2.5-5%, 3-5%, 3-7%, or 4-6% (w/v) of an amino acid or combination of amino acids. In some embodiments, two, three or more amino acids are present, such as selected from alanine, glycine, proline and/or arginine. In some embodiments, a composition contains about 0.5-4%, 1-4%, 1.5-4%, 2-4%, 2.5-4%, 3-4%, 3.5-4%, 0.5-1%, 0.5-1.5%, 0.5-2%, 0.5-2.5%, 0.5-3%, 0.5-3.5%, 0.5-4%,1-3%, 1-2%, 2-3%, or 1.5-2.5% (w/v) alanine or arginine. In some embodiments, a composition contains about 2%, 1.75%, 2.25%, 2.5%, 2.75%, 3%, 3.25%, 3.5%, 3.75% or 4% (w/v) alanine or arginine. In some embodiments, a composition contains about 6-12%, 7-12%, 8-12%, 9-12%, 10-12%, 11-12%, 6-11%, 6-10%, 6-9%, 6-8%, 6-7%, 7-11%, 8-10%, 7-10%, 11-9%, 7-8%, 8-9% or 9-10% (w/v) glycine. In some embodiment, a composition contains about 6%, 7%, 8%, 9%, 10%, 11% or 12% (w/v) glycine. In some embodiments, the recited concentration of amino acid is prior to freezing and/or lyophilization.

[0084] In some embodiments, a formulation does not comprise an amino acid(s) solution or does not contain an amino acid(s), other than the amino acids that are part of the polypeptide antigen.

[0085] In some embodiments, the formulation further comprises one or more additional ingredients. For example, the formulation may include one or more salts, such as sodium chloride, sodium phosphate, or a combination thereof. In general, each salt is present in the formulation at about 10 mM to about 200 mM.

[0086] The vaccine formulations may contain a buffer such as 20 mM TRIS-HCL. The pH of the formulation may also vary. In general, it is between about pH 6.2 to about pH 8.0. In one embodiment, the pH of the vaccine is about 7.4.

[0087] In another embodiment, the formulation further comprises a sugar alcohol such as sorbitol. In one embodiment, the formulation comprises 0.25% sorbitol.

[0088] In some embodiments, compositions and vaccine formulations of the invention may contain additional adjuvants, for instance, ImmunoStimulatory Sequences (ISS, CpG), and calcium phosphate. For ISS, protein samples are generally used at a final protein concentration 50 μg/ml. Other non-limiting examples of adjuvants include, but are not limited to,: CGP7909 (*e.g.*, see US Patent No. 7,223,741, which is herein incorporated by reference in its entirety), CpG1018 (see, for instance, US 2010/0183675, which is herein incorporated by reference in its entirety), Glucopyranosyl Lipid Adjuvant (GLA), PolyI PolyC (PIPC), N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2- (1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19840A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/ TWEEN 80 emulsion.

[0089] The invention includes compositions comprising the following formulations: 0.15 mg/mL antigen, 1.5 mg/mL aluminium, 20% trehalose, 2% alanine and 0.025% surfactant; 0.5 mg/mL antigen, 5 mg/mL aluminium, 20% trehalose, 2% alanine and 0.025% surfactant; 0.5 mg/mL antigen, 5 mg/mL aluminium, 20% trehalose, 1% sucrose, 2% alanine and 0.025% surfactant; and 0.5 mg/mL antigen, 5 mg/mL aluminium, 20% trehalose, 2% alanine and 0.025% surfactant. In some embodiments the antigens and/or surfactants in the these formulations are PA and Tween ® 80, respectively. In some embodiments, a composition of the invention comprises 5 mM NaPi, pH 7.0 buffer or 20mM Tris, pH 7.4. Other compositions included in the invention are described in the Examples.

[0090] Vaccines of the invention can be prepared for use as injectables. The composition can be a liquid formulation that is temperature stable (*e.g.*, can withstand a freeze/thaw cycle) or a frozen composition. The composition may also be used to produce a lyophilized dry powder vaccine which can be reconstituted, *e.g.*, with a pharmaceutically acceptable carrier prior to administration. Vaccine administration is generally by conventional routes, for instance, intravenous, subcutaneous, intraperitoneal, or mucosal routes. The administration may be by parenteral injection, for example, a subcutaneous or intramuscular injection.

[0091] In some embodiments, a composition of the invention is subjected to freezing and followed by sublimation under vacuum to produce a lyophilized composition.

[0092] The term "reconstituted" or "reconstitution" refers to the restoration of a lyophilized form to a liquid form, e.g., by rehydration, of a substance previously altered for preservation and/or storage, e.g., the restoration to a liquid state of a lyophilized rPA formulation of the application that has been stored. A lyophilized composition of the present application can be reconstituted in any aqueous solution which produces a stable, aqueous solution suitable for administration. Such an aqueous solution includes, but is not limited to, sterile water, TE (Tris EDTA), phosphate buffered saline (PBS), Tris buffer or normal saline. A lyophilized sample can be reconstituted with a lower, the same or higher volume than was used to lyophilize the sample.

[0093] It should be understood that a dose of a reconstituted lyophilized vaccine formulation of the application can be determined in light of various relevant factors including the conditions to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom, and can be administrated in a single dose, divided dose or multiple doses.

[0094] The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5 μg to 500 μg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. In one embodiment, the vaccine comprises at least about 10 μg PA, 25 μg PA, 50 μg PA, 75 μg PA, 100 μg PA, 125 μg PA, 150 μg PA, 200 μg PA, 225 μg PA, 250 μg, 275 μg, 300 μg PA. Precise amounts of antigen are dependent on the antigen to be delivered.

[0095] The vaccine may be given in a single dose schedule, or optionally in a multiple dose schedule. The vaccine composition may be administered, for instance, in a 0.5 mL dose. For pre-exposure prophylaxis, a multiple dose schedule is one in which a primary course of vaccination may be with 1-6 separate doses, followed by other doses given at subsequent time intervals to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months.

[0096] For post-exposure prophylaxis, the vaccine may also be administered according to a single dose or multiple dose regimen. For instance, in one embodiment, the vaccine is administered in 3 doses at times 0, 2 and 4 weeks post exposure. The dosage regimen will

also, at least in part, be determined by the need of the individual, upon the judgment of the practitioner and/ or upon results of testing, *e.g.*, measuring levels of immune response to the vaccine/antigen(s) such as antibody levels and/or T-cell activity against the antigen(s).

[0097] In addition, the vaccine containing the immunogenic antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, immunoglobulins, antibiotics, interleukins (e.g., IL-2, IL-12), and/or cytokines (e.g., IFN-beta, IFN-alpha).

[0098] In one embodiment, the vaccine is administered to a subject post-exposure to anthrax. In this embodiment, the vaccine may be administered in conjunction with an antibiotic. Antibiotics that may be administered with the vaccine include, but are not limited to, penicillin, doxycycline and ciprofloxacin.

[0099] The invention includes methods of treating (post-exposure prophylaxis) or preventing (pre-exposure prophylaxis) an anthrax infection comprising administering to a subject a pharmaceutically effective amount of a vaccine of the invention. In one embodiment, the anthrax infection is the result of inhaling anthrax (inhalation anthrax). As used herein, a pharmaceutically effective amount of a vaccine is an amount that induces an immune response. In one embodiment, a pharmaceutically effective amount of a vaccine is an amount comprising at least 25 μg PA. As used herein, a subject is a mammal such as a human.

[0100] The invention also provides methods of stimulating an immune response in a subject by administering to the subject an amount of a vaccine of the invention sufficient to stimulate an immune response. In one embodiment, immune stimulation is measured by increases in antibody titer that is specific for the antigen in the vaccine. In still other embodiments, immune stimulation is measured by an increased frequency in cytotoxic T lymphocytes specific for the antigen in the vaccine.

[0101] Also provided are methods of vaccinating a subject against a pathogen comprising administering a composition of the invention. Additionally provided are methods of vaccinating a subject against a pathogen comprising administering to a subject a pharmaceutical composition reconstituted from a lyophilized composition of the invention. The invention further includes methods of producing potent, alum based frozen vaccines comprising suspending a composition comprising at least about 10%, at least about 15%, at least about 20%, at least about 21%, at least about 25% or at least about 30% sugar and an

antigen adsorbed to an aluminum adjuvant and freezing said composition at a rate sufficient to freeze the suspended composition before sedimentation occurs, *e.g.*, flash freezing.

[0102] Some embodiments of the invention provide methods of preparing a stable lyophilized composition, comprising lyophilizing a composition of the invention, wherein the stability of the reconstituted lyophilized composition is measured by microphage lysis assay (MLA), size exclusion chromatography (SEC-HPLC) and/or anion exchange chromatography (AEX-HPLC).

[0103] For anthrax vaccines, the immunogenicity of the formulations can be tested as described in the various examples. For example, mice can be immunized with, for example, 10 μg, 20 μg, or more of rPA suspended in an adjuvant emulsion. Control mice are immunized with saline emulsified in adjuvant for use as negative controls. The mice are generally immunized, then bled at various intervals, e.g., day 0, day 21 and day 28 post-immunization. The serum is then analyzed for the presence of specific antibody, e.g., by ELISA, which can also be used to determine the titer of the antisera.

[0104] A mouse toxin-neutralizing antibody assay can also be used to determine if the anthrax vaccine formulations elicit protective antibodies. In this assay, mice immunized with rPA are then challenged i.p. with 2 lethal doses of lethal toxin (PA and lethal factor (LF)). Four days after challenge, the mice are scored for survivors.

[0105] The rPA formulations can also be used to prepare compositions comprising neutralizing antibodies that immunoreact with the anthrax toxin. The resulting antisera can be used for the manufacture of a medicament for treating exposure to anthrax. In one embodiment of the invention, the antibody composition comprises a purified anti-PA antibody. By "purified," it is meant that the antibody is substantially free of other biological material with which it is naturally associated. Purified antibodies of the invention are at least 60% weight pure, at least 70% weight pure, at least 80% weight pure, at least 90% weight pure or at least 95% weight pure. The antisera, or antibodies purified from the antisera, can also be used as diagnostic agents to detect either PA fragments or native protein.

[0106] The frozen and lyophilized formulations of the invention can be manufactured with increased potency by increasing the freeze rate. In one embodiment the formulation is flash frozen.

[0107] Potency can also be increased by freezing suspended rather than settled compositions. Compositions can be suspended by gentle shaking and immediately frozen.

[0108] The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention and in no way limiting.

Example 1: Freeze/Thaw of Liquid rPA and AVA Vaccines with and without Trehalose

[0109] rPA102 vaccine formulations with and without trehalose were prepared as outlined in **Table 1** below.

Table 1. Trehalose Formulations for Freeze/Thaw Assay

Sample	rPA (mg/ml)	ALHYDROGEL (mg/ml)	Buffer	pH	Trehalose	Arginine	TWEEN 80
rPA Control 1	0.15	1.5	20 mM TRIS-HCl	7.4	-	-	-
rPA Test 1					20%	2%	0.025%

[0110] After compounding, each sample was divided into two 8 ml aliquots in 10 ml glass tubes. For each sample, after gentle mixing overnight, one tube was placed at -80° C and the other tube was placed at 2-8° C after gentle mixing overnight.

[0111] Samples stored at -80° C overnight were thawed on the lab bench the next day for several hours (>3-4 hours) before observation and comparison to the 2-8° C samples that were brought to room temperature. Samples were photographed and total liquid height and ALHYDROGEL (aluminum hydroxide) height were measured. Regular rPA102 vaccine was compared before and after freeze/thaw. **Figure 1** is a photograph comparing rPA Control 1 sample at 2-8° C (labeled 5° C) to the -80° C sample after thaw. The photograph shows significant collapse of the alum gel in the rPA Control 1 sample subjected to freeze/thaw conditions. The level of sugar in a regular formulation that protected rPA102 from freeze/thaw stress was tested. As shown in Figure 1, freezing damaged rPA102 vaccine, and the potency (MRPT) data correlated with physio-chemical and gel height (collapsed). **Figure 2** is a photograph comparing rPA Test 1 sample at 2-8° C (labeled 5° C) to the -80° C sample

after thaw. There is no noticeable collapse of the alum in the thawed rPA Test 1 sample. **Table 2** provides an overview of the relative % alum height.

Table 2. Relative % Alum Height

Formulation	5° C	-80° C
rPA Control 1	100%	32.6%
rPA Test 1	100%	101.5%

[0112] A similar freeze/thaw experiment was performed with a test composition containing 15% trehalose, 0.15 mg rPA/mL, 2% alanine, 0.025% polysorbate 80, 25 mM NaPi, pH 7.4 compared to a control formulation without 15% trehalose with similar result (data not shown).

[0113] A vial of BioThrax® (Anthrax Vaccine Adsorbed), AVA, and a vial AVA + 25% trehalose were placed in -80° C after gentle mixing. A second vial of BioThrax and a second vial of AVA + 25% trehalose were placed at 2°-8° C overnight after gentle mixing. The next day, the -80° C vials were allowed to thaw and all vials were inspected. The aluminum gel height appeared to be about the same for the BioThrax stored at 2°- 8° C and the two AVA samples containing 25% trehalose. The aluminum gel height was much lower for BioThrax stored at -80° C (no trehalose). (Data not shown).

Example 2: Freeze/Thaw of Liquid rPA Vaccines with and without Sucrose

[0114] rPA102 vaccine formulations with and without sucrose were prepared as outlined in **Table 3** below.

Table 3. Sucrose Formulations for Freeze/Thaw Assay

Sample	rPA (mg/ml)	ALHYDROGEL (mg/ml)	Buffer	pH	Sucrose
rPA Control 2	0.5	5	20 mM TRIS-HCL	7.4	-
rPA Test 2					10%

[0115] After compounding, each sample was divided in to two 10 ml aliquots in 10 ml glass tubes. For each sample, one tube was placed at -80° C after gentle mixing overnight and the other tube was placed at 2-8° C after gentle mixing overnight.

[0116] Samples stored at -80° C overnight were thawed on the lab bench the next day for 2-3 hours before observations were made. Figure 3 contains a photograph of each formulation from 2-8° C (labeled 5° C) and -80° C after both being brought to room temperature. As shown, gel collapse occurred in both -80° C samples (rPA Control 2 and rPA Test 2) after thaw as compared to the samples that remained refrigerated at 2-8° C. However, the amount of gel collapse was visibly greater in the rPA Control 2 sample that did not include sucrose.

Example 3: In Vivo Mouse Potency Assay

[0117] Lyophilized vaccines were prepared as outlined in **Table 4**. Dried vaccines were reconstituted with water for injection to a final rPA concentration of 0.15 mg/ml (75µg/0.5 ml dose) and then diluted in normal saline by 10-fold to yield a dose level of 0.1 (DL).

[0118] Female CD-1 mice at 5-8 weeks of age and weighing 20-25 grams each were used for this study. The 0.1 DL of the vaccine was injected (0.5 ml) IP into groups of 20 female CD-1 mice and sera were collected on day 28 for the assessment of their ability to neutralize anthrax LT cytotoxicity in the toxin neutralization assay (TNA) in mice.

Table 4. Lyophilized Formulations for Mouse Potency Assay

Samples	Formulation
Lyophilized #1	10% trehalose, 0.5 mg/ml rPA, 5.0mg/ml aluminum, 0.25% sorbitol, 75 mM NaCl, 1% arginine, 20 mM Tris-HCL, pH 7
Lyophilized #2	No sugar, 0.15 mg/ml rPA, 1.5 mg/ml aluminum, 20 mM Tris-HCl, pH 7.4
Lyophilized #3	30% trehalose, 0.15 mg/ml rPA, 1.5 mg/ml aluminum, 2% arginine, 0.025% polysorbate 80, 20 mM Tris-HCl, pH 7.4
Lyophilized #4	20% trehalose, 0.15 mg/ml rPA, 1.5 mg/ml aluminum, 10% glycine, 0.025% polysorbate 80, 20 mM Tris-HCl, pH 7.4

[0119] The immunogenicity of the rPA102 formulation was investigated by calculating Neutralization factor (NF50). The neutralization factor, NF50 is defined as follows:

$$NF50 = \frac{ED50_{sample}}{ED50_{reference}}$$

[0120] Where effective dose 50% (ED50) reference standard was prepared by using a qualified serum reference standard stored at or below -20° C.

[0121] The geometric mean (GeoMean) of the NF50 of each vaccine formulation was calculated based on 20 NF50 values from 20 mice. T-test or one-way ANOVA were used to compare the geometric mean of NF50 of each formulation at the 95% confidence level. If the p value of the geometric mean NF50 was larger than 0.05, there was no significant difference in NF50 (potency) among the formulations. If the p value was less than 0.05, the geometric mean NF50 of the formulations was significantly different from each other.

[0122] The control (lyophilized #2) was an rPA102 formulation without stabilizer (0.15 mg/ml rPA, 1.5 mg/ml aluminum, 20 mM Tris-HCL, pH 7.4). The effect of freezing on the GeoMean NF50 values from the relative mouse potency assay for the regular rPA102 formulation is shown in **Figure 4**. The control formulation was susceptible to freeze/thaw damage with immunogenicity dropping significantly after the freezing process (**Figure 4**). The drop in immunogenicity corresponded to the decrease in the height of the aluminum gel in the solution.

[0123] Lyophilized Samples #1 and 2 showed significantly lower immunogenic potency relative to Lyophilized Samples #3 and 4 (which contained 30% and 20% trehalose, respectively). The effect of sugar in the formulation on lyophilization of rPA102 vaccine was shown by GeoMean NF50 results in **Figure 7**. Lyophilized Sample #1 (10% sugar) was not able to protect rPA102 from freeze-dry stress (see **Figure 7**). These results showed that 20% and 30% sugar was able to protect rPA102 from lyophilization stress. The appearance of gel collapse correlated with potency loss.

[0124] NF50 responses were determined from two more formulations that differ only by the absence or presence of trehalose: #1) 0.15mg/mL rPA, 1.5mg/mL alum, 2% arginine, 0.025% TWEEN 80 (polysorbate 80), 20mM Tris-HCL, pH 7.4 and #2 & #3) 0.15mg/mL rPA, 1.5mg/mL alum, 20% trehalose, 2% arginine, 0.025% TWEEN 80 (polysorbate 80), 20mM Tris-HCL, pH 7.4 and the effect of sugar before freezing on the GeoMean values for rPA102 is shown in **Figure 5**. In **Figure 5** formulation groups #2 & #3 are just different vials of the same formulation. Adding trehalose had no impact on the immunogenicity of the formulation as demonstrated by no statistical change in NF50 for the two formulations (3 samples), with and without sugar and no freezing (**Figure 5**). A comparison of GeoMean

values before and after freezing of these trehalose containing formulations is shown in **Figure 6**. There was no statistically significant change in immunogenicity (NF50) before and after freezing when this formulation was used (**Figure 6**). In addition, there was no collapse of the alum gel (gel height maintained) after freezing with this formulation (photographs in **Figure 6**). These results show that the tested formulations with 20% trehalose protected this rPA vaccine from freeze/thaw stress.

Example 4: Rabbit Immunogenicity and Stability Study

[0125] The immunogenicity of recombinant protective antigen (rPA) lyophilized vaccine formulations stored at 5 and 50 °C for 4 month was compared to Anthrax Vaccine Adsorbed (AVA) (BioThrax®) using a toxin neutralizing antibody assay (TNA) in New Zealand White (NZW) rabbits. This rabbit immunogenicity study utilized a two immunization schedule (Day 0 and Day 28) and bleeds were taken on days -1 or 0, 14, 21, 28, 35, 42, 56 and 70.

[0126] The rPA lyophilized vaccine was prepared using the ingredients shown in **Table 5**. The ingredients of the final formulation were blended prior to lyophilization and after reconstitution as shown in **Table 5**. Briefly, 2 mL of the suspension was filled in a 10 mL glass vial. The lyophilization was performed using a VirTis AdVantage lyophilizer. After lyophilization, the vaccines were stored at 5 and 50 °C.

Table 5. rPA Formulation

Ingredients	Pre-lyophilization (2 mL suspension in 10 vial)	Post reconstitution by adding 6.11 mL water to final volume of 6.67 mL
rPA, mg/mL	0.5	0.15
Aluminum, mg/mL	5	1.50
%Trehalose	25%	7.5%
Sorbitol	0.25%	0.075%
TWEEN 80	0.03%	0.0075%
Arginine	1%	0.30%
NaCl, mM	75	22.5
Tris-HCl, mM pH 7.4	20	6.0
Volume, mL	2	6.67

[0127] In particular, stock solutions were prepared and (except for TWEEN 80 and NaCl) pH adjusted to 7.4 using 0.1N NaOH and/or 0.1N HCl. After stock solutions were prepared, 150 mL of the following formulation blend was prepared in a 200 mL Nalgene bottle: 0.5

mg/mL rPA, 5.0 mg/mL aluminum, 30% Trehalose (w/v), 0.25% Sorbitol (w/v), 1% Arginine (w/v), 0.025% TWEEN 80, 75mm NaCl, 20mM Tris-HCl, pH 7.4, which was used to fill 10 mL vials with 2 mL of formulation blend.

[0128] After filling vials, the samples were dried using a VirTis AdVantage lyophilizer with the following program:

Initial freezing:

Step	Process	Setting
Freeze	Freeze (°C)	-60
	Additional Freeze time (min)	0
	Condenser (°C)	-80
	Vacuum (mTorr)	90

Drying:

Step	Temp °C	Time (min)	R/H	Vac mTorr
1	-60	120	H	90
2	-28	60	R	
3	-28	1250	H	
4	-28	550	H	
5	25	480	R	
6	25	600	H	
7	30	120	R	
8	30	300	H	
9	35	120	R	
10	35	300	H	
11	40	120	R	
12	40	300	H	
13	45	120	R	
14	45	255	H	

Post-drying:

Secondary Dry Set-Point Temperature	+65 °C
Post-Heat Settings	
Temperature (°C)	+25
Time (min)	1250
Vacuum (mTorr)	1250

[0129] Vials were stored as described in **Table 6**. On the day of immunization, 6.11 mL sterile water for injection was added to each vial to reconstitute the lyophilized samples. The vials were mixed end over end until all formulation components were completely dissolved. Dilutions (1:4, 1:16 and 1:64) of each test and control article were prepared in sterile normal saline.

[0130] NZW rabbits were used for the present study. NZW rabbits are commonly used as an animal model for *Bacillus anthracis* disease to test for toxicity, immunogenicity and efficacy studies, and NZW rabbits are considered to be a well-characterized model since they have similar pathogenesis and clinical presentation as seen in humans (EK Leffel et al., Clin Vaccine Immunol. 19(18):1158-1164, 2012; AJ Phipps et al., Microbiol Mol Biol Rev. 68(4):617-29, 2004). Each group of NZW rabbit (10 per vaccine group) received a 0.5 mL intramuscular injection with the 1:4, 1:16 & 1:64 dilutions of a lyophilized rPA vaccine formulation or AVA on days 0 and 28. AVA (BioThrax®) is a liquid anthrax vaccine that includes the 83kDa protective antigen protein and is formulated with 1.2 mg/mL aluminum (added as aluminum hydroxide in 0.85% sodium chloride), 25 mg/mL benzethonium chloride and 100 mg/mL formaldehyde (added as preservatives).

[0131] Serum samples were collected at days -1 or 0, 14, 21, 28, 35, 42, 56 and prior to termination on day 70. The TNA assay was performed by using serum collected on day -1 or 0, 14, 35 and 42. **Table 6** summarizes the study design.

Table 6. Rabbit Immunogenicity Study Design

Group #	Tested Vaccine	Vaccine Dilution	Immunization Schedule (Study Days)	Dose Volume (mL)	Blood Collection*	# of Animals (Rabbits)
1	AVA (positive control)	1:4	Day 0 and 28	0.5 mL	Prior to the day of dose initiation (Day -1 or 0) and on Days 14, 21, 28, 35, 42, 56 and 70	10 (5M/5F)
2		1:16				
3		1:64				
10	rPA Lyophilization 5°C, 4 months	1:4				
11		1:16				
12		1:64				
13	rPA Lyophilization 50°C, 4 months	1:4				
14		1:16				
15		1:64				
	*Serum from Days -1 or 0, 14, 35 and 42 tested					

[0132] The TNA assay is a functional test that evaluates the amount of antibody needed to inactivate the lethal B. anthracis toxin complex of LF and PA (lethal toxin, LT). The ability of test serum samples to neutralize lethal toxin in vitro was compared with that of a standard serum sample by using cytotoxicity as the endpoint of the assay (PR Pittman et al., Vaccine 24(17):3654-60, 2006).

[0133] Briefly, J774A.1 cells were cultured in flasks for 48 to 72 h in Dulbecco's modified Eagle media (DMEM) containing 4.5 g/liter d-glucose and supplemented with 10% heat-inactivated bovine serum, 2 mM L-glutamine, 1mM sodium pyruvate, penicillin (50 U/ml), streptomycin (50 µg/ml), and 0.11mM sodium bicarbonate. Cells were harvested and seeded in 96-well tissue culture plates at 30,000 cells/well, followed by 16 to 24 hour incubation. Serum samples were prepared in a separate 96-well microtiter plate at 2-fold dilutions for a total of seven dilutions per sample. The serum samples were then incubated with a constant concentration of LT (100 ng/ml PA and 80 ng/ml LF) for 1 hour. Then the serum sample with LT was added to the corresponding wells of the tissue culture plate containing the cells and incubated for four hours, after which 25 µl/well of 5 mg/ml of a tetrazolium salt, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), was added. After a 1-hour incubation, the cells were lysed by using 100 µl/well of acidified isopropanol (50% N, N-Dimethylformamide (with deionized water) and 20% SDS (200g in 1

liter of 50% dimethylformamide)) with the pH adjusted to 4.7 using HCl. Assay plates were incubated for an additional 16-24 hour, absorbance was measured at 570 and 690 nm in which 690 optical density values were subtracted from 570 values using a calibrated Molecular Devices VersaMax Plate Reader. The ED50, which is the dose that produces a quantal effect in 50% of the population in optical density measurement, was determined using SoftMax Pro software (version 5.4.1, Sunnyvale, CA).

[0134] ED50 for the mouse reference serum (Lot # MS011211) was used to determine the NF50 values of the test serum samples and the positive control (NF50 = ED50 Test / ED50 reference). The mouse reference standard was prepared by immunizing 300 mice with AVA vaccine containing CPG 7909 adjuvant. The serum from the 300 mice was collected, pooled and stored frozen at -80 °C as the reference standard. **Tables 7A-C** show a comparison of the average kinetic data (NF50) for lyophilized rPA stored at 5 °C, lyophilized rPA stored at 50 °C, and AVA control stored at 5 °C from serum samples at 14, 35 and 42 days, respectively.

Table 7A. Comparison of average kinetic data (NF50) at Day 14

Sample	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA
Storage	5°C	50°C	5°C	5°C	50°C	5°C	5°C	50°C	5°C
Dilution	1:4	1:4	1:4	1:16	1:16	1:16	1:64	1:64	1:64
Avg	0.10	0.13	0.05	NRS	NRS	NRS	0.02	0.02	NRS
Stdev	0.07	0.08	0.02	NRS	NRS	NRS	0.01	0.01	NRS
CV%	0.72	61%	50%	NRS	NRS	NRS	31%	26%	NRS
GeoMean	0.07	0.11	0.04	NRS	NRS	NRS	0.02	0.02	NRS

Table 7B. Comparison of average kinetic data (NF50) at Day 35

Sample	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA
Storage	5°C	50°C	5°C	5°C	50°C	5°C	5°C	50°C	5°C
Dilution	1:4	1:4	1:4	1:16	1:16	1:16	1:64	1:64	1:64
Avg	3.69	3.25	1.80	1.86	1.28	0.79	0.26	0.14	0.06
Stdev	2.19	1.47	0.85	0.82	0.99	0.46	0.25	0.11	NRS
CV%	59%	45%	47%	44%	78%	59%	96%	77%	0%
GeoMean	3.14	2.98	1.61	1.70	0.94	0.67	0.16	0.10	0.06

Table 7C. Comparison of average kinetic data (NF50) at Day 42

Sample	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA	rPA lyo	rPA lyo	AVA
Storage	5°C	50°C	5°C	5°C	50°C	5°C	5°C	50°C	5°C
Dilution	1:4	1:4	1:4	1:16	1:16	1:16	1:64	1:64	1:64
Avg	2.54	2.35	1.15	1.21	0.78	0.46	0.17	0.12	0.05
Stdev	1.74	1.18	0.60	0.67	0.60	0.26	0.17	0.08	0.04
CV%	68%	50%	52%	56%	77%	56%	97%	64%	75%
GeoMean	2.07	2.13	1.01	1.08	0.57	0.40	0.11	0.10	0.04

NRS = Non-responders

[0135] All animals tested negative for toxin neutralizing activity at day 0. <NF50> reached maximum levels at 35 days for all three tested vaccines at all three dose levels

(**Figure 8A-B, 9A-B, and 10A-B**), showing that rPA had similar or better immunogenicity kinetic as AVA at all three doses. As shown in **Figure 8A-B**, lyophilized rPA (1:4 dilution) stored at both 5 and 50 °C had a higher <NF50> than AVA (1:4 dilution). Similarly, at dilutions of 1:16 and 1:64, the lyophilized rPA had higher <NF50> than the comparable AVA dilution (see **Figures 9A-B and 10A-B**).

[0136] For the 1:4 dilutions at 35 days, the <NF50>geomean (gm) for lyophilized rPA stored at 5 and 50 °C was found to be 3.14 and 2.98, respectively; and the <NF50>gm of AVA at 1:4 dilution was 1.61. There was no statistical differences in <NF50>gm between the lyophilized rPA formulations (1:4) stored at 5 °C versus 50 °C, p=0.82 (t.test). The <NF50>gm of the combined data (rPA lyo 5 and 50°C) was found to be 3.06; and the combined <NF50>gm was statistical higher than that of the AVA reference (1.61), p = 0.034 (t.test). At day 42 (1:4), there was no statistical different in <NF50>gm between rPA lyo stored at 5 versus 50 °C (2.07 and 2.13, respectively), p=0.92 (t.test); and the combined <NF50>gm was found to be 2.10. The combined <NF50>gm of 2.10 was statistically higher than that of AVA reference (1.01), p = 0.028 (t.test).

[0137] For the 1:16 dilutions at day 35, the <NF50>gm for lyophilized rPA stored at 5 and 50 °C was found to be 1.70 and 0.94, respectively. There was no statistical differences in <NF50>gm between rPA lyo stored at 5 and 50 °C, p=0.081 (t.test). The combined <NF50>gm was found to be 1.27, and there was no statistical difference in combined <NF50>gm for rPA lyo (5 and 50°C) and that of AVA reference (0.67), p = 0.064 (t.test). At day 42 (1:16), there was no statistical different in <NF50>gm between rPA lyo stored at 5 versus 50 °C (1.08 and 0.57, respectively), p=0.071 (t.test); and the combined <NF50>gm was found to be 0.78. The combined <NF50>gm of 0.78 was statistical higher than that of AVA reference (0.40), p = 0.05 (t.test).

[0138] For the 1:64 dilutions at day 35, the <NF50>gm for lyophilized rPA stored at 5 and 50 °C were not statistically different (1.06 and 0.10, respectively), p=0.386 (t.test). The combined <NF50>gm was found to be 0.13 and the AVA reference was 0.06. At day 42 (1:64), there was no statistical different in <NF50>gm between rPA lyo stored at 5 and 50 °C (0.11 and 0.10, respectively), p=0.91 (t.test); and the combined <NF50>gm was found to be 0.11. There was no statistical difference between the combined <NF50>gm of 0.11 and the AVA reference (0.04), p = 0.35 (t.test).

[0139] Geometric mean of NF50 (<NF50>gm) of lyophilized rPA stored at 5 and 50 °C compared to AVA at three dilutions (1:4, 1:16, and 1:64) at days 35 and 42 are shown in **Figures 11A-B, 12A-B, and 13A-B**.

[0140] At day 35, the <NF50>gm for the lyophilized rPA vaccine stored at 50 °C was found to be 2.98, 0.94 and 0.1 for doses 1:4, 1:16 and 1:64, respectively. The <NF50>gm of the lyophilized rPA vaccine stored at 5 °C were similar at day 35 (3.14, 1.7 and 0.16, respectively) with no statistically significant difference compared to 50 °C (alph=0.05). Similar results were found at day 42. These data demonstrated that the immunogenicity of the lyophilized rPA vaccine stored at 50 °C for 4 months was not significantly different from the lyophilized rPA vaccine stored at 5 °C.

[0141] At day 35, the combined <NF50>gm (5 °C and 50 °C) was found to be 3.06, 1.27 and 0.1 at doses 1:4, 1:16 and 1:64, respectively; and the p values for the combined <NF50>gm compared to AVA at 1:4 and 1:16 were p=0.034 and p=0.064, respectively. The combined <NF50>gm was found to be non-inferior (statistical higher or no difference) to that of the AVA. Similar results are shown for day 42. At 42 days, the combined <NF50> values for 1:4, 1:16, and 1:64 were 2.10, 0.78, 0.11, respectively; and the p values for the combined <NF50>gm compared to AVA at 1:4, 1:16, and 1:64 were p=0.92, p=0.071, and p=0.91, respectively. These immunogenicity data show that the rPA lyophilized vaccine stored at 5 °C and 50 °C for 4 months was at least as immunogenic as the AVA vaccine.

[0142] In sum, these results show that the tested lyophilized formulation was capable of stabilizing the rPA vaccine for at least 4 months at 50 °C. The data demonstrated that the rPA lyophilized formulation had superior thermal stability profile compared to AVA vaccine. Thus, the results showed that the rPA lyophilized formulation was effective for rPA anthrax vaccine storage, and the tested formulation was room temperature stable and able to circumvent a cold chain distribution.

Example 5: Guinea Pig Immunogenicity Study

[0143] A guinea pig immunogenicity study is outlined in **Table 8**.

Table 8. Guinea Pig Immunogenicity Study Design

Vaccine	Pre-dilution	Dilution	GP Required(n) 315 to 385 g	Reported Results
AVA Reference Lot	None	1/1.6	6 male + 6 female (12)	Survivors / Total
		1/4	6 male + 6 female (12)	Survivors / Total

		1/10	6 male + 6 female (12)	Survivors / Total
		1/25	6 male + 6 female (12)	Survivors / Total
rPA102 Fresh	None	1/1.6	6 male + 6 female (12)	Survivors / Total
		1/4	6 male + 6 female (12)	Survivors / Total
		1/10	6 male + 6 female (12)	Survivors / Total
		1/25	6 male + 6 female (12)	Survivors / Total
rPA102 Liquid 12-15 months at 5°C	None	1/1.6	6 male + 6 female (12)	Survivors / Total
		1/4	6 male + 6 female (12)	Survivors / Total
		1/10	6 male + 6 female (12)	Survivors / Total
		1/25	6 male + 6 female (12)	Survivors / Total
rPA102 Lyophilized 1 month at 40°C (then reconstituted)	None	1/1.6	6 male + 6 female (12)	Survivors / Total
		1/4	6 male + 6 female (12)	Survivors / Total
		1/10	6 male + 6 female (12)	Survivors / Total
		1/25	6 male + 6 female (12)	Survivors / Total
Challenge Preparation				
Colony Forming Units per 0.1 mL dose	40		4 male + 4 female (8)	Deaths/Total
Total number of vaccinated animals			200 (100 males and 100 females)	

Example 6: Immunogenicity and Physiochemical Stability in Mice

[0144] Three lyophilized rPA vaccine formulations were tested for immunogenicity and physiochemical stability compared to liquid rPA vaccine. The content and purity of rPA vaccines (physiochemical stability properties) were measured by macrophage lysis assay (MLA), size exclusion chromatography (SEC-HPLC), and anion exchange chromatography (AEX-HPLC). The immunogenicity of the rPA vaccines was evaluated by vaccinating CD-1 mice with four dose levels and testing the ability of the mice serum to neutralize anthrax toxin (NF50).

Liquid rPA Vaccine Formulation

[0145] Liquid rPA formulation (F1) was prepared under aseptic condition at 0.15 mg/mL rPA, 1.5 mg/mL alum, 2% Alanine, 0.01% TWEEN 80, 25 mM NaPi, pH 7.0. The samples for the stability assays contained 5 mL liquid suspension filled in 10 mL glass vial (10 doses per vial). The intended human dose is 75 μg rPA/750 μg alum per 0.5 mL with intramuscular injection.

Lyophilization rPA Vaccine Formulations

[0146] Three lyophilized rPA formulations were prepared. The final formulations, prior to lyophilization, are shown in **Table 9**. The first lot (lyoA) was formulated with 0.15 mg/mL rPA, 1.5 mg/mL alum, 20% Trehalose, 2% Ala, 0.025% Tw80 and 5 mM NaPi at pH 7.0. The second and third lots (lyoB and lyoC, respectively) contained 3.3x higher rPA and alum concentrations (0.5 mg/mL and 5.0 mg/mL, respectively) with slight variations in sugar, amino acid and buffer as indicated in **Table 9**.

Table 9. Concentration of Final Formulation Blended Prior to Lyophilization

Lot #	rPA (mg/mL)	Alum (mg/mL)	Sugar	Amino Acid	TWEEN 80	Buffer	Liq-Lyo Vol, mL
LyoA	0.15	1.5	20% Trehalose	2% Alanine	0.025%	5mM NaPi, pH 7.0	2mL fill in 10mL vials
LyoB	0.5	5	20% Trehalose	2% Alanine	0.025%	5mM NaPi, pH 7.0	2mL fill in 10mL vials
LyoC	0.5	5	20% Trehalose + 1% Sucrose	2% Glycine	0.025%	20mM Tris, pH 7.4	2mL fill in 10mL vials

[0147] All three rPA lyophilized formulations were designed such that most rPA protein was bound to alum with little or no free rPA protein in the solution. 2 mL liquid suspension was filled into a 10 mL glass vial. Lyophilization was performed using FTS LyoStar® II with the following processing parameters.

Initial freezing:

Step	Process	Setting
Freeze	Freeze (°C)	-60
	Additional Freeze time (min)	0
	Condenser (°C)	-80
	Vacuum (mTorr)	90

Drying:

Step	Temp °C	Time (min)	R/H	Vac mTorr
1	-60	120	H	90
2	-28	60	R	
3	-28	1250	H	
4	-28	550	H	

5	25	480	R	
6	25	600	H	
7	30	120	R	
8	30	300	H	
9	35	120	R	
10	35	300	H	
11	40	120	R	
12	40	300	H	
13	45	120	R	
14	45	255	H	

Post-drying:

Secondary Dry Set-Point Temperature	+65 °C
Post-Heat Settings	
Temperature (°C)	+25
Time (min)	1250
Vacuum (mTorr)	1250

[0148] Prior to vaccination and testing, the lyophilized samples were reconstituted with water for injection (WFI) to produce a final concentration of 0.15 mg/mL rPA and 1.5 mg/mL alum for all three formulations. The final concentration was accomplished by adding 1.55, 6.2 and 6.18 mL of WFI to vials of the first lot (LyoA), second lot (LyoB) and third lot (LyoC), respectively. The final suspension volume per vial for LyoA was 2.0 mL and for both lots LyoB and LyoC were 6.7 mL. The concentration of rPA vaccines after reconstitution are shown in **Table 10**.

Table 10. Concentration of lyophilized rPA vaccine after reconstitution

Lot #	rPA (mg/mL)	Alum (mg/mL)	Sugar	Amino Acid	TWEEN 80	NaCl	Buffer	Recons WFI Vol, mL	Final Vol, mL	# Doses/vial (0.5mL/dose)
Lyo A	0.15	1.5	20% Trehalose	2% Alanine	0.025%	-	5mM NaPi, pH 7.0	1.55	2.0	4.0
Lyo B	0.15	1.5	6% Trehalose	0.6% Alanine	0.075%	-	1.5mM NaPi, pH 7.0	6.2	6.7	13.3
Lyo C	0.15	1.5	6% Trehalose + 0.3% Sucrose	0.6% Glycine	0.075%	-	6mM Tris, pH 7.4	6.18	6.7	13.3

[0149] The total number of doses per vial in lots LyoB and LyoC were higher than that of lot LyoA (13.3 vs 4.0). The manufacturing cost of a higher doses vial (such as 13.3 doses per vial) is significantly lower than that of a lower dose vial (e.g., 4 doses per vial). Thus, it was

an economical preference to develop a formulation and process for producing higher dose vials.

Stability & Test Assays

[0150] The rPA liquid formulation (F1) was placed in a long term stability program at storage temperatures of 5, 25 and 40 °C. The three rPA lyophilized lots (LyoA, LyoB, and LyoC) were placed in a long term stability program at storage temperatures of 5, 25, 40, and 50 °C. The physiochemical tests were performed for both the liquid (F1) and lyophilized rPA vaccine formulations. Four (4) months of stability data for the samples were collected.

[0151] The physiochemical properties of the rPA test vaccines were evaluated by a series of assays, i.e., MLA, SEC-HPLC and AEX-HPLC. All of these assays were performed using rPA proteins extracted from the alum. The extraction procedure utilized 200 mM potassium phosphate/0.01% Tw80/0.9% NaCl.

[0152] A rPA bulk drug substance (BDS) stored at -80 °C was used as a reference control. The BDS control was purified from a ΔSterne-1(pPA102)CR4 strain of *B. anthracis*, which was developed by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) as an asporogenic, non-toxigenic expression system for the production of rPA. The rPA BDS was purified and stored in 20 mM Tris, 0.9% NaCl, pH 7 under -80 °C frozen condition.

I. Macrophage Lysis Assay (MLA)

[0153] The *in vitro* macrophage lysis assay (MLA) was used to determine the cytotoxicity of rPA on the murine macrophage cell line J7774A.1. The MLA measures the activity of rPA and rLF toxin. The assay involves adding rPA protein to lethal factor protein (rLF) to form a lethal toxin complex, which caused pore formation with the cell membranes of the macrophages, leading to cell lysis.

[0154] The activity of the rPA lyophilized vaccines (using rPA desorbed from alum) was measured relative to a BDS reference standard and reported as a percentage of the reference standard. The percentage of cells surviving toxin challenge was determined. For example, 100% MLA activity of rPA vaccine would indicate there was no loss in the cytotoxicity activity of rPA adsorbed to alum and after lyophilized. In brief, microphage cells were seeded in a 96 well plate at $5x10^4$ cells/well and the plate was placed in a CO_2 incubator overnight. The next day, 100ul of serial diluted rPA test samples or rPA reference standard

(starting rPA concentration was 800ng/ml and then 1:2 diluted down to 0.8ng/mL) was mixed with rLF (the rLF concentration was constant at 100ng/ml) and added to the wells. Four hours later, 25ul of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5mg/mL was added to each well and the plate was incubated for 1.5 hours. After the 1.5 hour incubation, 100ul of soluble solution (20% SDS in 50% dimethylformamide solution) was added to the wells and the plate was incubated at 37°C overnight. The next day, the plate was read by a plate reader at 570nm and OD readings were graphed with 4-parameter model using software (SoftMax). The ED50 value of the test sample rPA was then compared to that of the rPA reference standard, and their ratio was used to report relative activity of the rPA test sample. The accuracy of the assay was determined to be +/-30%.

II. Size-Exclusion Chromatography (SEC-HPLC)

[0155] SEC-HPLC is a chromatographic technique used to separate proteins based on their molecular weight and size and it is commonly used to assess the stability of a protein in a formulation. A larger protein typically has a shorter retention time (elute out sooner) in a SEC-column than a smaller protein. Protein that is degraded (or fragmented) will become smaller in size and elute out of the chromatography later. Vice versa, aggregated protein will elute sooner. For example, an aggregated rPA protein that has increased in size would have a shorter retention time than the native rPA protein, and a degraded rPA protein (breakdown in size) would have a longer retention time.

[0156] SEC-HPLC is a common assay used to assess the physiochemical stability of a protein in a formulation. Compared to MLA, the SEC-HPLC is, in general, relatively more sensitive and quantitative with precision of less than 5% in %peak area and less than 0.1% in retention time within the same run.

[0157] The SEC-HPLC was performed using a TSK G3000SWXL column (Tosoh BioScience P/N M1182-05M) with a mobile phase of 50 mM sodium phosphate/250 mM potassium chloride at pH 7.4. An ultraviolet (UV) detector at 215 nm or fluorescence detector at 280 nm (excitation) and 335 nm (emission) were used for the detection.

III. Anion Exchange Chromatography (AEX-HPLC)

[0158] AEX-HPLC separates proteins based on their net electrostatic charge. In general, the assay involved injecting rPA protein solution into an HPLC equipped with an anion exchange column. The rPA protein was retained by the anion exchange (AEX) column

(stationary phase) due to electrostatic interaction. The rPA protein was then eluted out by using mobile phase with gradually increased ionic strength (NaCl concentration). The ionic strength (or elution time) at which the rPA molecules eluted is related to its net charges.

[0159] The rPA molecule is known to be susceptible to degradation by deamidation mechanism (D'Souza, Journal of Pharmaceutical Sciences, 102(2):454-461, 2013) resulting in a change in net change. Deamidation increases the negative charge of the rPA protein due to the conversion of asparagine residues to aspartate (more negative). The deamidated rPA (typically referred to as the acidic species) elutes in higher ionic strength solution and has a longer elution time than the native rPA protein. The purity of the rPA was characterized by the %main peak.

[0160] The AEX-HPLC was performed using Hamilton PRP-X500 Anion Exchange Column (P/N 79641) using mobile phase A of 25 mM Tris at pH 8.0 and mobile phase B of 25 mM Tris, 0.5 M NaCl at pH 8.0. Similar to SEC-HPLC, UV or fluorescence detectors were used.

IV. Immunogenicity Test

[0161] Immunogenicity was evaluated using toxin neutralizing assay (TNA) (Hering et al., Biologicals 32 (2004) 17-27; Omland et al., Clinical and Vaccine Immunology (2008) 946-953; and Li et al., Journal of Immunological Methods (2008) 333:89-106. ED50 values for the mouse reference serum (prepared as described above) were used to determine the NF50 values of the test serum sample and the positive control. The first month stability data are reported herein.

[0162] For the *in vivo* immunogenicity test, 20 female CD-1 mice per group received a single 0.5 mL intraperitoneal (i.p.) injection of LyoA, LyoB, LyoC, or liquid rPA (F1). Four dilution dose levels were evaluated (1:4, 1:8, 1:16 and 1:32). The dilutions were prepared on the day of the vaccination with saline solution. Serum samples were obtained by cardiac bleeding at Day 28 after vaccination.

Physiochemical Stability Results

[0163] Table 11 summarizes the MLA, SEC-HPLC & AEX-HPLC results of the three lyophilized formulations stored at 4 months at 5, 25, 40 and 50 °C and the BDS reference control.

Table 11. Summary of physiochemical stability data at 4 months

4 months physiochemical stability data				
Name	Temp, °C	MLA %Relative to Reference	SEC %Purity, Main Peak	AEX %Purity, Main Peak
Ref BDS	-80	100	84.0	80.8
lyoA	5	116	85.8	87.1
	25	102	85.5	86.6
	40	107	83.1	84.7
lyoB	5	111	84.9	87.8
	25	112	84.9	86.2
	50	108	84.5	85.3
lyoC	5	117	84.7	87.5
	25	123	84.8	84.5
	50	104	84.7	70.5
Ref BDS	-80	100	98.6	78.2
Liq. rPA F1, 1 month	5	98	95.3	65.5
	25	65	91.4	25.6
	40	0	0	0

Macrophage Lysis Assay Results

[0164] These MLA results show that there was no significant drop in rPA cytotoxicity for the three lyophilization formulations stored at 4 months at temperatures up to 40 and 50 °C when compared to the BDS reference control (within error variability of +/- 30%).

[0165] In comparison, there is a significant drop in MLA value for the liquid rPA vaccine (F1) stored for one month at 5, 25 and 40 °C. The MLA values were found to be 98%, 65% and 0% at 5, 25 and 40 °C, respectively. **Figure 14** shows a comparison of the MLA% as a function of storage temperature for the three lyophilized lots stored for 4 months versus the rPA liquid lot (F1) stored for 1 month. These results show that the three lyophilized formulations maintained the MLA activity of the rPA vaccine for at least up to 4 months at 40 and 50 °C, whereas the liquid rPA vaccine loss all it MLA activity after storage for 1 month at 40 °C.

Size-Exclusion Chromatography (SEC-HPLC) Results

[0166] A typical SEC chromatograph of the rPA reference control (BDS) is shown in **Figure 15B**. The percent peak area was calculated for each sample peak. The native rPA

protein (monomer) eluted at 17.1 minutes with a % main peak area of 85%. The second peak eluted at 18.2 minutes and corresponds to the aggregated rPA molecule main peak area of ~15%. The %purity of rPA protein was determined by the % area of the 17.1 minutes elution peak. i.e., the purity of the rPA protein (%purity rPA) = the percent peak area of the rPA peak (corresponding to monomer rPA with retention time of ~17.1 min) / the total peak area).

[0167] The purity results for the LyoA, LyoB and LyoC were comparable to the purity results for the rPA reference BDS of 84.0% stored at -80 °C, see **Table 11**. The SEC-HPLC data showed that there was no significant drop in purity compared to the reference control for all three lyophilized formulations stored at 4 months at all storage temperatures.

[0168] As a comparison, there was a significant drop in purity for the liquid rPA vaccine when stored at an accelerated temperature (25, 40, or 50 °C) for 1 month. The relative % purity of rPA of the liquid formulation dropped -3.3%, -7.2% and -98.6% at 5, 25 and 40 °C, respectively.

[0169] The %purity of the BDS reference control were found to be 84.0% and 98.6% for the lyophilized and the liquid assays, respectively. The two tests were performed at different times. The difference in % purity of the reference control was not unusual. The difference could be due to varying SEC-column condition and sample preparation procedure. The relative % purity (over reference control) is typically used to compare the stability samples at different times and across different laboratories.

[0170] **Figure 15A** shows the relative decrease of rPA SEC % purity (over BDS reference control) as a function of storage temperature for the three lyophilized formulation compared to the liquid formulation. The data demonstrated there was no change in SEC-purity for the lyophilized rPA vaccine stored for at least 4 months at 40 or 50 °C, whereas the liquid rPA vaccine was completely degraded after 1 month storage at 40 °C.

Anion Exchange Chromatography (AEX-HPLC) Results

[0171] A typical AEX chromatograph for the rPA reference control (BDS) is shown in **Figure 16B**. The purity of rPA was characterized by the %main peak area. The native rPA protein eluted at 21.0 minutes with a main peak of ~81.4%. The deamidated rPA (typically referred to acidic species) eluted between 21.7 and 22.4 minutes with an area of 16.6%. The %main peak area was calculated by first integrating the area under the curve of all peaks (except the buffer peak) and then the %main peak area corresponds to the rPA peak with ~retention time of 21.0 min, (i.e., %main peak area = peak area (RT=21.0 min) / Sum of all

peak areas). Similar to SEC-HPLC, the purity of rPA was the same as %main peak area. The accuracy of the AEX assay was about 10-15%.

[0172] The AEX %purity of the first lyophilized lot (lyoA) stored for 4 months was found to be 87.1, 86.6, 84.7 at 5, 25 and 40°C, respectively. The AEX purity for the second lyophilized lot (lyoB) was 87.8%, 86.2% and 85.3% at 5, 25 and 50°C, respectively. The AEX %purity for the third lyophilized lot (lyoC) was 87.5%, 84.5% and 70.5% at 5, 25 and 50°C, respectively. These purity results for LyoA, LyoB, and LyoC were comparable to the purity of the rPA reference control (BDS) of 80.8%. There is no significant drop in purities over the reference control for all three lyophilized formulations after 4 months storage at all storage temperatures.

[0173] The AEX purity of the liquid rPA vaccine stored at 1 month was found to be 65.5%, 25.6% and 0% at 5, 25 and 40 °C, respectively; and the AEX purity for rPA reference control (BDS) was 78.2% when used in the assay testing liquid rPA. The AEX purity results are summarized in **Table 11.**

[0174] As shown in **Figure 16A**, there was a significant drop in the AEX-purity in the rPA liquid formulation (F1) at 5, 25 & 40 °C compared to the lyophilized formulations. The AEX-data demonstrated that there was no loss in purity of the lyophilized rPA vaccine stored for at least 4 months at 40 or 50 °C; whereas, the liquid vaccine was completely degraded at 1 month after storage at 40 °C.

In vivo Immunogenicity Results

[0175] The NF50 results in mice at dose levels 0.25, 0.125, 0.0625 and 0.03125 for the three lyophilized formulations stored for 1 month each were determined. **Figures 17A-D, 18A-D, and 19A-D** compares the NF50 data (n=20) across various temperatures (5, 25 and 40 °C) at their corresponding formulation and dose level.

[0176] The geometric mean of the NF50 (<NF50>gm) at 5, 25 and 40 °C for the four doses level of the three lyophilization formulations are summarized in **Tables 12A-C**.

Tables 12A-C. GeoMean of NF50 (n=20) for the three lyophilized formulations at 5, 25 & 40 °C at four dose levels (0.25, 0.125, 0.0625 & 0.03125)

13A. <NF50> gm of **Lyo A** stored at 5, 25 and 40 °C at one month at four dose levels				
Dose Level	5 °C	25 °C	40 °C	Are the <NF> gm significantly different among temperatures? (p<0.050) ANOVA Test

0.25	1.12	1.20	1.13	No, p = 0.97
0.125	0.84	0.86	0.50	No, p = 0.10
0.0625	0.33	0.28	0.39	No, p = 0.36
0.03125	0.10	0.15	0.13	No, p = 0.18

13B. <NF50> gm of **Lyo B** stored at 5, 25, 40 and 50 °C at one month at four dose levels				
Dose Level	5 °C	25 °C	40 °C	Are the <NF> gm significantly different among temperatures? (p<0.050) ANOVA Test
0.25	1.16	0.81	0.86	No, p = 0.22
0.125	0.33	0.46	0.32	No, p = 0.20
0.0625	0.12	0.15	0.13	No, p = 0.59
0.03125	0.05	0.06	0.05	No, p = 0.78

13C. <NF50> gm of 31 **Lyo C** stored at 5, 25, 40 and 50 °C at one month at four dose levels				
Dose Level	5 °C	25 °C	40 °C	Are the <NF> gm significantly different among temperatures? (p<0.050) ANOVA Test
0.25	0.98	0.80	1.02	No, p = 0.37
0.125	0.43	0.45	0.46	No, p = 0.94
0.0625	0.13	0.15	0.20	No, p = 0.21
0.03125	0.07	0.06	0.07	No, p = 0.78

[0177] For lot LyoA after storage for 1 month, the <NF50>gm were found to be 1.12, 1.20, and 1.13 at 5, 25 and 40 °C respectively at dose level 0.25. There was no statistically significant difference in the <NF50>gm among the three storage temperatures (5, 25 and 40 °C) at p=0.97. Similarly, it was also shown that there was no statistical difference in <NF50>gm among the various storage temperatures at the other tested dose levels (0.125, 0.0625 and 0.01315) for all three lyophilized formulations (LyoA, LyoB, and LyoC). The NF50 data demonstrated that there was no significant drop in immunogenicity for the three lyophilized formulations at 1 month up to 40 °C.

[0178] In contrast, the liquid rPA formulation (F1) showed a significant drop in immunogenicity at the 25 and 40 °C storage temperatures at 1 month. **Table 13** shows the <NF50>gm results for the rPA liquid formulation stored at 1 month at 5, 25 and 40 °C.

Table 13. Geomean of NF50 of liquid rPA formulation F1 stored at 1 month at 5, 25 & 40 °C at four dose levels (0.3, 0.2, 0.1 & 0.05)

<NF50> gm of Liquid rPA (F1) formulation stored at 5, 25 and 40 °C at one month at four dose levels				
Dose Level	5 °C	25 °C	40 °C	Are the <NF> gm significantly different among temperatures? (p<0.050) ANOVA Test
0.3	1.26	0.69	0.30	Yes, p = 0.0023
0.2	1.18	0.55	0.13	Yes, p = <0.0001
0.1	1.03	0.22	0.09	Yes, p = <0.0001
0.05	0.32	0.15	0.03	Yes, p = 0.0010

[0179] At the 0.3 dose level, the <NF50>gm was found to be 1.26, 0.69 and 0.30 at 5, 25 and 40 °C, respectively, and there was a statistically significant difference in all the <NF50>gm at p=0.0023. Similarly, at the lower dose levels of 0.2, 0.1 and 0.05, the <NF50>gm significantly dropped as the storage temperature increases (see **Figure 20A-D**). The NF50 was found to decease significantly when the liquid vaccine was stored at 25 °C as compare to 5 °C at all four dose levels, and progressively more at 40 °C.

[0180] In sum, the immunogenicity data demonstrated the superiority of the lyophilized rPA formulations over the liquid rPA formulation. The lyophilized formulations maintain their immunogenicity at 25 and 40 °C for at least 1 month, while the immunogenicity of the liquid formulation decreases significantly over similar storage conditions.

[0181] Like most liquid vaccines, liquid rPA vaccines were found to be unstable at accelerated storage temperatures (e.g., 25 and 40 °C). Liquid rPA vaccine lost its immunogenicity and key physiochemical properties when stored at 40 °C for 1 month. Similarly, the key physiochemical properties of the vaccine were also significantly degraded. The content and purity of vaccine as measured by macrophage lysis assay (MLA), size exclusion chromatography (SEC-HPLC) and anion exchange chromatography (AEX-HPLC) were found to significantly decrease at 25 °C and be undetectable at 40 °C for 1 month. Liquid rPA vaccine was known to be susceptible to deamidation reaction especially when it was adsorbed on aluminum and stored at accelerated temperature.

[0182] Three lyophilized formulations were manufactured as lot number: lyoA, lyoB and lyoC. These three new rPA lyophilization formulations had superior stability profile over the rPA liquid vaccines. There was no statistically significant change in purity in all key physiochemical assays (MLA, SEC-HPLC and AEX-HPLC) for all three lots of lyophilized formulations when stored at 4 month for temperatures up to 50 °C. In addition, there is no significant drop in immunogenicity (NF50) when the lyophilized vaccines were stored at 1 month for up to 40 °C at four dose levels.

[0183] The results herein show that the lyophilized rPA formulations had superior physiochemical and immunological stability profiles over the liquid rPA formulation. The lyophilized formulations maintained all the key physiochemical properties tested by MLA, SEC and AEX at storage temperatures up to 50 °C and over the 4 month storage time period. The lyophilized formulations also maintained immunogenicity at storage temperatures up to 40 °C for at least 1 month. On the contrary, the liquid rPA formulation showed a complete loss of the physiochemical properties (MLA, SEC, and AEX) and a significant drop in immunogenicity after storage for 1 month at 40 °C.

Example 7: Formulations and Lyophilization with Other Adjuvants

[0184] CPG 7909 Bulk Drug Substance (BDS) is packaged as a lyophilized powder in high density polyethylene (HDPE) bottles, heat sealed in multi-layer (mylar, foil) pouches, and stored at -20°C ± 5°C.

[0185] Glucopyranosyl Lipid Adjuvant (GLA) was obtained from Avanti Polar Lipids, Inc. It was packaged in 2 mL amber glass vials containing 25 mg of lyophilized GLA powder and stored at -20°C ± 5°C. GLA is described in Arias et al. (2012) PLoS ONE 7(7):e41144.

[0186] Bulk Drug Substance (BDS): 2.81 mg/mL rPA, 0.9% NaCl and buffered in 20 mM Tris-HCl at pH 7.4 was used. It was stored at -80 °C and thawed at 5 °C overnight prior to use.

[0187] PolyI PolyC (PIPC) was obtained from InviviGen in 20 mL glass vials as a lyophilized cake containing 50 mg of PIPC. It was stored at 5 °C.

Table 14. Chemicals and Source

Chemical Name	Source
Tris Hydrochloride, Ultrapure	Amresco
2% ALHYDROGEL (10 mg/mL	Brenntag

aluminum)	
α,α-Trehalose dihydrate	Hayashibara Biochemicals
Sodium phosphate, monobasic, Anhydrous	Sigma Aldrich
Sodium phosphate, dibasic, hepta-hydrate	BDH
Polysorbate 80, N.F.	J.T. Baker
L-Alanine	EMD

Table 15. Equipment/Materials

Name	Source
VirTis AdVantage Plus Lyophilizer	SP Scientific
Wheaton Serum Vials, Borosilicated Glass	VWR
Slotted Rubber Stoppers for Lyo Vials	VWR
Flip-Off Crimp Seals	VWR

Stock Solution Preparations

[0188] Two 60% (w/v) solutions of trehalose were prepared in 20mM Tris and 5mM NaPi buffers, separately, and were sterile filtered. A 10% (v/v) solution of Polysorbate80 was prepared in DI water and then sterile filtered. Two 12% (w/v) solutions of alanine were prepared in 20mM Tris and 5mM NaPi buffers, separately, and were sterile filtered.

[0189] One aluminum hydroxide stock solution was buffered by adding 7 mL of 1M Tris buffer, pH 7.4, to 343 mL of 2% AlOH (or 10 mg/mL aluminum) and was titrated to pH 7.4. A second aluminum hydroxide stock solution was buffered by adding 1.75 mL of 1M NaPi buffer, pH 7.0, to 348.25 mL of 2% AlOH and was titrated to pH 7.0. The dilution effect from the buffer addition and subsequent titration was not accounted for in either preparation.

Adjuvant Preparations

[0190] GLA adjuvant was prepared in 20mM Tris-HCl buffer, pH 7.4 by adding 31.2 mg of powder into a 50 mL conical tube and adding 15.6 mL of buffer. The mixture was sonicated for a total of 60 seconds in 10 second intervals with 10 second rests in between to a maximum power of 15 W. A turbid mixture was obtained of 2 mg/mL GLA.

[0191] CPG 7909 stocks were prepared in 20mM Tris, pH 7.4 or 5mM NaPi, pH 7.0 buffers. In each preparation, about 200 mg of CPG 7909 powder were fully dissolved in a final volume of 10 mL. A clear solution was obtained for both preparations.

[0192] A 2 mg/mL stock solution of PIPC was prepared by dissolving a 50 mg lyophilized cake of PIPC in 25 mL of 5 mM NaPi buffer, pH 7.0. The mixture was sonicated for a total of 60 seconds in 10 second intervals with 10 second rests in between to a maximum power of 15 W. A clear solution with no visible particles was obtained.

Formulation Procedure for Blending

[0193] **Table 16** shows the chemical composition of the prepared formulations:

Table 16: Formulation Blends for Liquid and Lyophilization

Sample nos.	Study Group	Chemical Composition										NaCl (residual) (mM)
		rPA (mg/mL)	Alum (mg/mL)	Trehalose (%)	Tween 80 (%)	Adjuvant (mg/mL)	Alanine (%)	Tris-HCl Buffer (mM)	NaPi Buffer (mM)	pH	Adjuvant Name	
7-13	CPG-Liq	0.15	1.5	0	0.03	0.50	0	20	0	7.4	CPG	8
14-20	CPG-Lyo	0.15	1.5	20	0.03	0.50	2	20	0	7.4	CPG	8
34-39	GLA-Liq	0.15	1.5	0	0.03	0.50	0	20	0	7.4	GLA	8
40-45	GLA-Lyo	0.15	1.5	20	0.03	0.50	2	20	0	7.4	GLA	8
73-75	PIPC/CPG-Liq	0.15	1.5	0	0.03	0.50	0	1.1	5	7.0	PIPC+CPG	8
76-81	PIPC/CPG-LYO	0.15	1.5	20	0.03	0.50	2	1.1	5	7.0	PIPC+CPG	8

[0194] The formulation blends in **Table 16** were prepared using buffered stock solutions as shown in **Table 17**.

Table 17: Stock/Excipient Volumes Used for Formulation Blend Preparations

Study Group	Formulation Volumes Added (mL) Stock Solution										Final Volume (mL)
	rPA Stock (mg/mL)	AlOH Stock (mg/mL)	Trehalose Stock (%)	Tween 80 Stock (%)	Alanine Stock (%)	CpG Stock (mg/mL)	GLA Stock (mg/mL)	Poly(IC) Stock (mg/mL)	NaPi Buffer (mM)	Tris Buffer (mM)	
	2.8	**10**	**60**	**10**	**12**	**20**	**2**	**2**	**5**	**20**	
CPG-Liq	0.86	2.40	0	0.040	0	0.40	0	0	0	12.30	16
CPG-Lyo	1.07	3.00	6.67	0.050	3.333	0.50	0	0	0	5.38	20
GLA-Liq	0.86	2.40	0	0.040	0	0	4.0	0	0	8.70	16
GLA-Lyo	1.07	3.00	6.67	0.050	3.333	-	5.0	0	0	0.88	20
PIPC/CPG-Liq	0.86	2.40	0	0.040	0	0.40	0	4.0	8.30	0	16
PIPC/CPG-LYO	1.07	3.00	6.67	0.050	3.333	0.50	0	5.0	0.38	0	20

[0195] The order of addition used for blending all excipients was as follows: aluminum hydroxide → trehalose → TWEEN 80 → alanine → buffer → rPA → CPG or GLA adjuvant

[0196] A final volume of 20 mL was prepared for samples 14-20, 40-45 and 76-81 for lyophilization. Blends were split into 10 mL glass vials in 2 mL aliquots and set aside for lyophilization.

Lyophilization Procedure

[0197] Samples were lyophilized using a VirTis Plus Freeze Dryer. A 73 hour cycle was employed and entered as described in **Table 18**.

Table 18: Lyophilization Cycle

#	Step	Temp (°C)	Time (Hr)	Ramp/ Hold	Vacuum (mTorr)
1	Thermal Treatment	-60	*fast*	-	-
2		-60	2	H	
3		-28	1	R	
4		-28	20	H	
5	Extra Freezing	-28	10	H	20
6	Primary Drying	25	8	R	20
7		25	10	H	
8		30	1	R	
9		30	5	H	
10	Secondary Drying	35	1	R	
11		35	5	H	
12		40	1	R	
13		40	4	H	
14		45	1	R	
15		45	4	H	
Post-Drying		25	-	-	3000

[0198]

Summary of Data

[0199] The immunological response of the six vaccines formulations was tested in mice (n=10) with one immunization using IP route. The serum was collected 28 days after immunization. TNA data at dose level = 0.4 was determined as described in Example 3 and the results are summarized in **Table 19**.

[0200] The mean NF50 was found to be 53.6, 48.9, 69.9, 64.7, 89.4 and 77.3 for CPG-liq, CPG-lyo, GLA-liq, GLA-lyo, PIPC/CPG-liq and PIPC/CPG-lyo samples, respectively. There is no statistical different in the mean NF50 of the liquid versus the lyophilized formulations for CPG, GLA and PIPC/CPG. The data demonstrated the lyophilized formulation and process is capable of maintaining the immunogenicity, even in the presence and of other adjuvants.

Table 19: NF50 of liquid versus lyophilization formulations of CPG, GLA and PIPC/CPG adjuvant containing vaccines

n	NF50 (DL =0.4)					
	CPG-Liq	CPG-Lyo	GLA-Liq	GLA-Lyo	PIPC/CPG-Liq	PIPC/CPG-Liq
1	51.2	43.1	114.4	108.9	91.6	62.8
2	106.3	29.2	115.2	23.7	100.8	67.2
3	103.9	77.4	43.1	65.1	114.5	119.2
4	33.1	17.2	50.3	60.9	74.6	84.0
5	10.3	47.8	122.0	83.3	19.4	63.2
6	37.3	31.4	55.9	29.2	249.5	68.6
7	47.6	51.8	50.1	115.8	61.0	84.8
8	54.7	66.4	33.3	79.3	65.3	62.4
9	42.8	36.5	81.7	67.9	29.8	79.4
10	48.9	87.9	32.5	12.9	87.4	81.2
Mean	53.6	48.9	69.9	64.7	89.4	77.3
Stdev	29.9	22.5	35.5	34.6	63.6	17.3
P value T-test	0.695		0.746		0.573	

n	Log NF50 (DL=0.4)					
	CPG-Liq	CPG-Lyo	GLA-Liq	GLA-Lyo	PIPC/CPG-Liq	PIPC/CPG-Liq
1	1.7	1.6	2.1	2.0	2.0	1.8
2	2.0	1.5	2.1	1.4	2.0	1.8
3	2.0	1.9	1.6	1.8	2.1	2.1
4	1.5	1.2	1.7	1.8	1.9	1.9
5	1.0	1.7	2.1	1.9	1.3	1.8
6	1.6	1.5	1.7	1.5	2.4	1.8
7	1.7	1.7	1.7	2.1	1.8	1.9
8	1.7	1.8	1.5	1.9	1.8	1.8
9	1.6	1.6	1.9	1.8	1.5	1.9
10	1.7	1.9	1.5	1.1	1.9	1.9
Mean Log	1.7	1.6	1.8	1.7	1.9	1.9
GeoMean	45.6	44.1	62.2	53.7	72.4	75.8
Stdev	0.3	0.2	0.2	0.3	0.3	0.1
P value T-test	0.898		0.607		0.849	

Example 8: Immunogenicity of Lyophilized rPA Vaccines

[0201] This Examples compares liquid vaccine freshly made vs lyophilized vaccine stored at 5 and 50 °C for 1 month and compares CPG formulations made in NaPi (pH 7.0) vs Citric (pH 5.5) buffers.

[0202] There are four formulation evaluated under this study:

rPA alum in NaPi (pH 7.0)

rPA alum + CPG in NaPi (pH 7.0)

rPA alum + CPG in Critic (pH 5.5)

rPA alum + GLA in Tris (pH 7.4)

Stock Solution Preparations

[0203] Three 60% (w/v) solutions of trehalose were prepared, one in 20mM Tris (pH 7.4), a second in 5mM NaPi buffers (pH 7.0), and a third in 20mM Na-Citrate (pH 5.5). A 10% (v/v) solution of Polysorbate80 was prepared by mixing 10 mL of concentrated Tween80 in 90 mL of DI water. Three 12% (w/v) solutions of alanine were prepared, one in 20mM Tris (pH 7.4), a second in 5mM NaPi buffers (pH 7.0), and a third in 20mM Na-Citrate (pH 5.5) and all were sterile filtered.

[0204] Three aluminum hydroxide stock solutions were buffered by adding 1M buffers into the 2% AlOH. The resulting stocks were titrated to the desired pH to match the buffer in use. The dilution effect from the buffer addition and subsequent titration was not accounted for in any of the preparations.

Table 19: Formulations Prior to Lyophilization

#	Buffer	Storage Condition	rPA mg/mL	Aluminum mg/mL	Trehal. %	Alanine %	CPG mg/mL	GLA mg/mL	TWEEN 80 (%)
1	5mM NaPI pH 7.0	Liquid Fresh	0.5	5.0	20.0	2.0	-	-	0.025
2		Lyo 5°C, 1 mo							
3		Lyo 50°C, 1 mo							
4	5mM NaPI pH 7.0	Liquid Fresh	0.45	4.5	20.0	2.0	1.5	-	0.025
5		Lyo 5°C, 1 mo							
6		Lyo 50°C, 1 mo							
7	Na-Citrate pH 5.5	Liquid Fresh	0.45	4.5	20.0	2.0	1.5	-	0.025
8		Lyo 5°C, 1 mo							
9		Lyo 50°C, 1 mo							
10	20mM Tris-HCL pH 7.4	Liquid Fresh	0.45	4.5	20.0	0.0	-	0.30	0.025
11		Lyo 5°C, 1 mo							
12		Lyo 50°C, 1 mo							

[0205] Each formulation was blended and then lyophilized at 2 mL per vial, using the lyophilization process as described in Example 7.

Table 20: Liquid Fresh or Reconstituted Concentrations

#	Buffer	Storage Condition	rPA mg/mL	Aluminum mg/mL	Trehal. %	Alanine %	CPG mg/mL	GLA mg/mL	TWEEN 80 (%)
1	5mM NaPI pH 7.0	Liquid Fresh	0.15	1.5	6.06	0.61	0	0	.008
2		Lyo 5°C, 1 mo							
3		Lyo 50°C, 1 mo							
4	5mM NaPI pH 7.0	Liquid Fresh	0.15	1.5	6.67	0.67	.5	0	.008
5		Lyo 5°C, 1 mo							
6		Lyo 50°C, 1 mo							

7	Na-Citrate pH 5.5	Liquid Fresh	0.15	1.5	6.67	0.67	.5	0	.008
8		Lyo 5°C, 1 mo							
9		Lyo 50°C, 1 mo							
10	20mM Tris-HCL pH 7.4	Liquid Fresh	0.15	1.5	6.67	0	0	0.1	.008
11		Lyo 5°C, 1 mo							
12		Lyo 50°C, 1 mo							

Animal model

[0206] Each animal receive 0.5 mL of 1/16 dilution of the listed formulations. 5Female/5Male Guinea pig per group (n=10). IM immunization was performed on Day 0 and Day 14. Blood collection was performed on day 14, 28 and 35. The TNA data at day 28 was analyzed and presented.

NF50 Data

[0207] Figure 21 shows the NF50 and the standard deviation of mean of the 12 formulations.

[0208] Table 21 shows the numerical values of each mouse of the 12 formulations.

Table 21: NF50 and LogNF50 For Each Mouse For Each of the 12 Formulations.

NF50 (DL=0.063)

n	rPA Alhydrogel NaPi Liq	rPA Alhydrogel NaPi 5°C	rPA Alhydrogel NaPi 50°C	250µg CpG NaPi Liq	250µg CpG NaPi 5°C	250µg CpG NaPi 50°C	250µg CpG Citrate Liq	250µg CpG Citrate 5°C	250µg CpG Citrate 50°C	50µg GLA Tris Liq	50µg GLA Tris 5°C	50µg GLA Tris 50°C
1	1.4	0.9	1.0	2.9	0.9	4.8	1.5	1.9	2.3	1.1	2.2	2.4
2	1.1	0.7	0.4	2.5	2.1	1.9	4.6	2.4	3.3	2.3	0.8	2.4
3	1.0	1.8	1.5	1.2	0.8	5.1	3.0	2.8	4.3	2.1	1.2	1.8
4	0.6	1.0	1.0	1.8	1.9	2.6	4.2	2.9	4.2	1.7	2.0	4.2
5	1.1	1.3	2.0	2.4	2.6	1.8	0.9	1.6	2.8	1.4	0.8	2.1
6	1.3	1.4	0.2	1.7	1.0	1.5	1.5	1.3	1.9	1.4	1.2	0.5
7	1.3	1.0	0.8	1.9	1.4	1.4	0.8	2.7	6.3	0.9	0.9	1.6
8	0.7	0.4	0.2	0.9	1.4	1.1	1.5	0.9	1.2	1.3	1.1	0.6
9	0.4	0.5	1.0	0.8	0.6	1.9	1.5	2.3	1.5	0.6	1.1	1.1
10	1.3	0.5	0.5	1.2	0.8	0.6	2.0	1.2	0.6	0.4	1.3	0.4
Mean	1.0	0.9	0.9	1.7	1.4	2.3	2.2	2.0	2.8	1.3	1.3	1.7
SD	0.3	0.5	0.6	0.7	0.7	1.5	1.4	0.7	1.7	0.6	0.5	1.2
%CV	33.9	49.1	66.6	40.8	48.1	65.8	62.6	36.0	61.1	46.4	38.6	67.5
P Value (ANOVA)	0.770			0.152		0.132	0.356			0.412		

Log NF50 (DL=0.063)

n	rPA Alhydrogel NaPi Liq	rPA Alhydrogel NaPi 5°C	rPA Alhydrogel NaPi 50°C	250µg CpG NaPi Liq	250µg CpG NaPi 5°C	250µg CpG NaPi 50°C	250µg CpG Citrate Liq	250µg CpG Citrate 5°C	250µg CpG Citrate 50°C	50µg GLA Tris Liq	50µg GLA Tris 5°C	50µg GLA Tris 50°C
1	0.15	-0.03	0.00	0.46	-0.03	0.68	0.19	0.28	0.36	0.03	0.35	0.38
2	0.04	-0.19	-0.44	0.39	0.31	0.29	0.67	0.38	0.52	0.36	-0.12	0.38
3	-0.02	0.26	0.17	0.09	-0.08	0.71	0.47	0.45	0.63	0.32	0.09	0.26
4	-0.20	-0.01	0.00	0.25	0.29	0.41	0.63	0.47	0.63	0.24	0.30	0.62
5	0.06	0.10	0.31	0.38	0.42	0.26	-0.07	0.22	0.45	0.13	-0.12	0.33
6	0.11	0.14	-0.74	0.24	0.01	0.19	0.19	0.12	0.27	0.16	0.09	-0.35
7	0.12	-0.02	-0.09	0.27	0.15	0.16	-0.12	0.43	0.80	-0.06	-0.03	0.21
8	-0.13	-0.41	-0.61	-0.05	0.13	0.04	0.19	-0.07	0.06	0.12	0.06	-0.22
9	-0.45	-0.34	0.01	-0.11	-0.21	0.27	0.19	0.37	0.19	-0.22	0.04	0.04
10	0.11	-0.30	-0.27	0.08	-0.11	-0.24	0.29	0.09	-0.23	-0.40	0.10	-0.36
Mean Log	-0.02	-0.08	-0.17	0.20	0.09	0.28	0.26	0.27	0.37	0.07	0.08	0.13
Geo Mean	0.95	0.83	0.68	1.58	1.23	1.89	1.83	1.88	2.33	1.17	1.19	1.34
SD	0.19	0.22	0.34	0.19	0.21	0.28	0.26	0.18	0.31	0.24	0.15	0.3
P Value (ANOVA)	0.472			0.201		0.210	0.603			0.847		

[0209] The NF50 data shows superior stability of the four lyophilized formulations. It also demonstrated the robustness of the formulations.

[0210] No statistical difference of NF50 mean and Geomean among liquid fresh, lyo (5 and 50 °C for 1 month) for all four formulations: (see Table 21)

[0211] No statistical difference of mean and geomean of NF50 between NaPi vs Citric buffer for rPA alum + CPG formulation. (see Table 21)

Example 9: Formulations with Influenza Antigen(s)

[0212] Formulations containing influenza antigen(s) are formulated using methods similar to the disclosed herein for rPA formulations, except for the presence of influenza antigen(s) and the absence of rPA antigens.

[0213] Examples of formulations containing influenza antigens are listed in **Table 20**.

Table 22. Examples of Formulations Containing Influenza Antigen for Lyophilization

Formulation #	Influenza Antigen mg/ml	Alum mg/ml	Sugar	Amino Acid	TWEEN 80	Liq. Lyoph. Vol.
1	0.15	1.5	20% Trehalose	none	0.025%	2 mL
2	0.15	1.5	30% Trehalose	none	0.025%	2 mL
3	0.15	1.5	20% Trehalose	2% Ala	0.025%	2 mL
4	0.15	1.5	30% Trehalose	2% Ala	0.025%	2 mL
5	0.15	1.5	20% Trehalose	2% Gly	0.025%	2 mL
6	0.15	1.5	30% Trehalose	2% Gly	0.025%	2 mL
7	0.15	1.5	20% Trehalose	2% Arg	0.025%	2 mL
8	0.15	1.5	30% Trehalose	2% Arg	0.025%	2 mL
9	0.15	1.5	10% Trehalose	2% Ala	0.025%	2 mL
10	0.15	1.5	10% Trehalose	2% Gly	0.025%	2 mL
11	0.15	1.5	10% Trehalose	2% Arg	0.025%	2 mL
12	0.5	5	20% Trehalose	none	0.025%	2 mL
13	0.5	5	30% Trehalose	none	0.025%	2 mL
14	0.5	5	20% Trehalose	2% Ala	0.025%	2 mL
15	0.5	5	30% Trehalose	2% Ala	0.025%	2 mL
16	0.5	5	20% Trehalose	2% Gly	0.025%	2 mL
17	0.5	5	30% Trehalose	2% Gly	0.025%	2 mL
18	0.5	5	20% Trehalose	2% Arg	0.025%	2 mL
19	0.5	5	30% Trehalose	2% Arg	0.025%	2 mL
20	0.5	5	10% Trehalose	2% Ala	0.025%	2 mL
21	0.5	5	10% Trehalose	2% Gly	0.025%	2 mL
22	0.5	5	10% Trehalose	2% Arg	0.025%	2 mL

[0214] Two sets of formulations are made, one in 5mM NaPi, pH 7.0 buffer or 20mM Tris, pH 7.4 buffer. The influenza antigen is an influenza hemagglutinin. Formulations may also contain another adjuvant such as CPG at 0.5 mg/mL, PIPC at 0.5 mg/mL, GLA at 0.1 mg/Ml or a combination thereof.

[0215] Each formulation is lyophilized, 2 mL per vial, using the lyophilization process as described in Example 7.

[0216] Each formulation is tested in immunogenicity studies, stability studies and/or efficacy studies.

WHAT IS CLAIMED IS:

1. A composition for preparation of a lyophilized vaccine comprising at least one antigen adsorbed to an aluminum adjuvant and at least 20% (w/v) non-reducing sugar.

2. A temperature stable liquid vaccine composition comprising at least one antigen adsorbed to an aluminum adjuvant and at least 20% (w/v) sugar.

3. A composition comprising, prior to lyophilization, at least one antigen absorbed to an aluminum adjuvant and at least 20% (w/v) non-reducing sugar, wherein after reconstitution the non-reducing sugar is at least 6% (w/v).

4. The composition of any one of claims 1-3, wherein the composition comprises a surfactant.

5. A composition comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant and at least 15% (w/v) sugar for preparation of a lyophilized vaccine.

6. A temperature stable liquid vaccine composition comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant and at least 15% (w/v) sugar.

7. The composition of any one of claims 1-6, wherein said composition further comprises an amino acid.

8. A composition for preparation of a lyophilized vaccine comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant, an amino acid and at least 10% (w/v) sugar.

9. A stable liquid vaccine composition comprising at least one antigen adsorbed to an aluminum adjuvant, a surfactant, an amino acid and at least 10% (w/v) sugar.

10. The composition of any one of claims 1-9, wherein the aluminum adjuvant is selected from the group consisting of aluminum phosphate and aluminum sulfate.

11. The composition of any one of claims 1-9, wherein the aluminum adjuvant is aluminum hydroxide.

12. The composition of claim 11, wherein the composition contains about 0.5 to 1.5 mg/ml aluminum hydroxide.

13. The composition of claim 12, wherein the composition contains about 0.5 mg/ml aluminum hydroxide.

14. The composition of claim 12, wherein the composition contains about 1.5 mg/ml aluminum hydroxide.

15. The composition of any one of claims 4-14, wherein the surfactant is selected from the group consisting of polysorbate 80 and polysorbate 20.

16. The composition of claim 15, wherein the surfactant is polysorbate 80.

17. The composition of any one of claims 4-16, wherein the composition contains about 0.020% or 0.025% (w/v) surfactant.

18. The composition of claims 1-17, wherein the sugar is a non-reducing sugar selected from the group consisting of trehalose, sucrose, and a combination thereof.

19. The composition of claims 18, wherein the sugar is trehalose.

20. The composition of claims 18, wherein the sugar is sucrose.

21. The composition of claim 18, wherein the sugar is trehalose and sucrose.

22. The composition of any one of claims 5-21, wherein the composition contains about 15-40% (w/v) sugar.

23. The composition of any one of claim 1-22, wherein the composition contains about 20-40% (w/v) sugar.

24. The composition of claim 23, wherein the composition contains about 20-35% (w/v) sugar.

25. The composition of claim 24, wherein the composition contains about 25-40% (w/v) sugar.

26. The composition of any one of claims 1-25, wherein the composition contains greater than about 20%, 21%, 22%, 23%, 24% and 25% (w/v) sugar.

27. The composition of any one of claims 1-26, wherein the antigen is an Anthrax antigen.

28. The composition of claim 27, wherein the Anthrax antigen is protective antigen.

29. The composition of claim 28, wherein the protective antigen is at least about 80% identity to the polypeptide of SEQ ID NO: 2.

30. The composition of any one of claims 28-29, wherein the composition comprises about 150-500 μg/ml protective antigen.

31. The composition of claim 30, wherein the composition comprises about 150, 175, 200, 225, 250, 275, 300, 325, 400, 375, 400, 425, 450, 475 or 500 μg/ml protective antigen

32. The composition of claim 31, wherein the Anthrax antigen is a cell-free filtrate from an avirulent *B. anthracis* strain.

33. The composition of claim 32, wherein the avirulent *B. anthracis* strain is V770-NP1-R.

34. The composition of any one of claims 7-33 wherein said amino acid is selected from the group consisting of arginine, alanine, proline, glycine, and any combination thereof.

35. The composition of claim 34, wherein the composition contains about 0.5-4% (w/v) alanine or arginine.

36. The composition of claim 40, wherein the composition contains about 2% (w/v) alanine or arginine.

37. The composition of claim 34, wherein the composition contains about 6-12% (w/v) glycine.

38. The composition of claim 37, wherein the composition contains about 10% (w/v) glycine.

39. The composition of any one of claims 1, 3-5, 7, 8, and 10-38, wherein said composition is subjected to sublimation under vacuum to produce a lyophilized composition.

40. A lyophilized composition, which is lyophilized from the composition of any one of claims 1, 3-5, 7, 8, and 10-38.

41. A reconstituted composition comprising the lyophilized composition of claim 39 or 40 reconstituted in an aqueous solution.

42. The reconstituted composition of claim 41, wherein the aqueous solution is selected from the group consisting water, Tris EDTA (TE), phosphate buffered saline (PBS), Tris buffer or saline.

43. The composition of any one of claims 1, 3-5, 7, 8, and 10-38, wherein the composition retains at least 80%, at least 90% or at least 95% purity after storage in lyophilized form for at least 4 months at 50 °C.

44. The composition of any one of claims 1, 3-5, 7, 8, and 10-38, wherein the composition retains at least 80%, at least 90% or at least 95% immunogenicity after storage in lyophilized form for at least 1 months at 40 °C.

45. A method of vaccinating a subject against a pathogen comprising administering the composition of any one of claims 1-44.

46. A method of vaccinating a subject against a pathogen comprising administering to a subject a pharmaceutical composition reconstituted from the lyophilized composition of claim 39 or 40.

47. A method of producing a potent, alum based frozen vaccine comprising suspending a composition comprising at least about 10% sugar and an antigen adsorbed to an aluminum adjuvant and freezing said composition at a rate sufficient to freeze the suspended composition before sedimentation occurs.

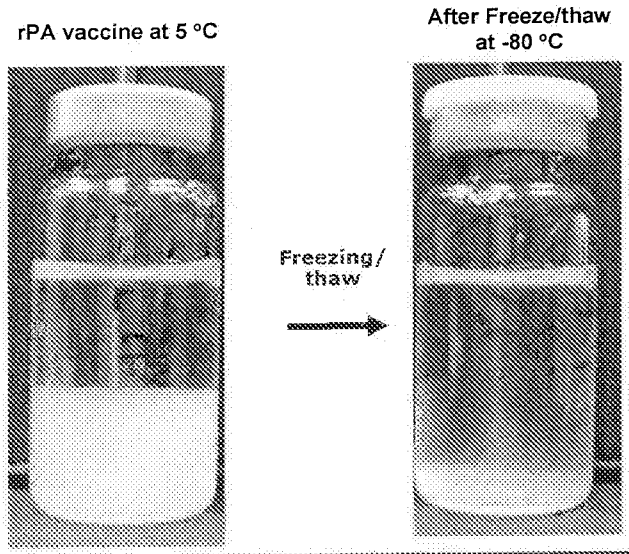
48. The method of claim 47, wherein said composition contains at least about 15% sugar.

49. The method of claim 47, wherein said composition contains at least about 20% sugar.

50. A method of preparing a stable lyophilized composition, comprising lyophilizing a composition of any one of claims 1, 3-5, 7, 8, and 10-38, wherein the stability of the reconstituted lyophilized composition is measured by microphage lysis assay (MLA), size

exclusion chromatography (SEC-HPLC) and/or anion exchange chromatography (AEX-HPLC).

Comparison of rPA102 vaccine before and after Freeze/Thaw



Regular Formulation
0.15mg/mL rPA, 1.5mg/mL Alum, 20mM Tris-HCl, pH 7.4

Freeze/Thaw	Intact rPA102		Desorbed rPA102		
	Fluorescence (320/350)	In-vivo (MRPT)**	MLA	RP-HPLC (Purity)	SE-HPLC (%Monomer)
Before	1.21	0.38	18%	82.84	94.58
After Freezing	0.94*	0.20***	2%	74.95	79.8

* Indicating change in rPA102 protein structure after freezing, data from AD (AD12117 FD samples)
** Data from MRPT group
*** Statistical significant at 95% CI

- **Freezing damages rPA102 vaccine**
- **Potency (MRPT) data correlates with physio-chemical and gel height (collapsed)**

FIG. 1

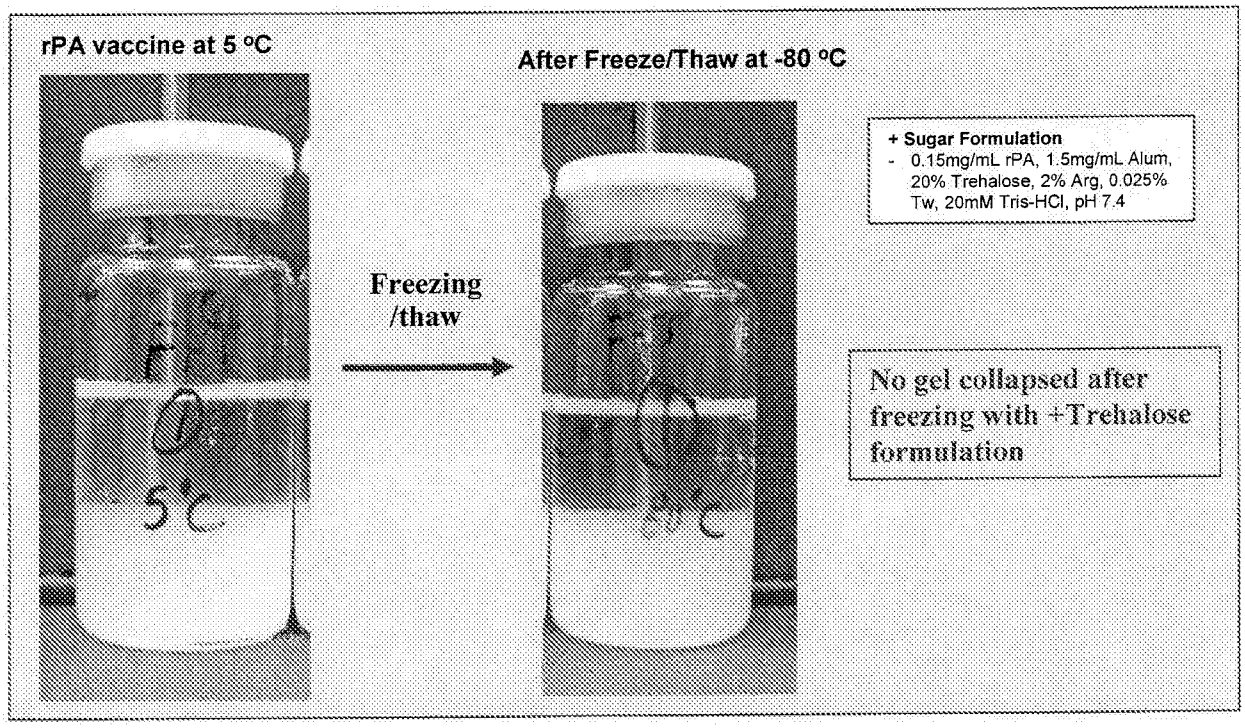

rPA vaccine at 5 °C
After Freeze/Thaw at -80 °C
Freezing
/thaw
+ Sugar Formulation
- 0.15mg/mL rPA, 1.5mg/mL Alum, 20% Trehalose, 2% Arg, 0.025% Tw, 20mM Tris-HCl, pH 7.4
No gel collapsed after freezing with +Trehalose formulation

FIG. 2

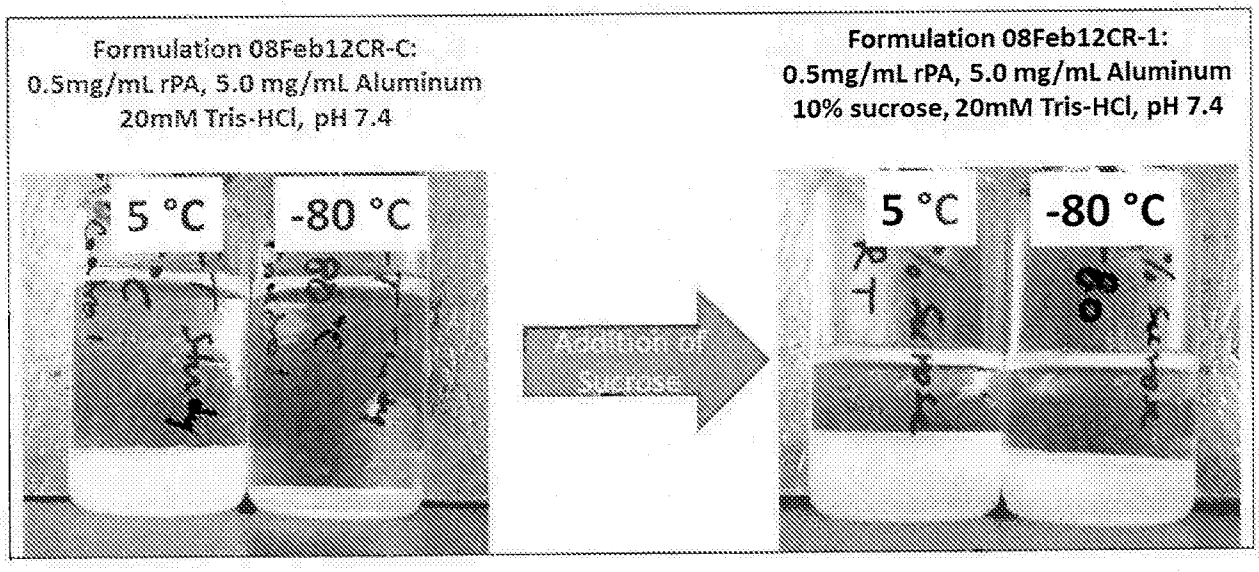

Formulation 08Feb12CR-C:
0.5mg/mL rPA, 5.0 mg/mL Aluminum
20mM Tris-HCl, pH 7.4
Formulation 08Feb12CR-1:
0.5mg/mL rPA, 5.0 mg/mL Aluminum
10% sucrose, 20mM Tris-HCl, pH 7.4
5 °C
-80 °C
5 °C
-80 °C

FIG. 3

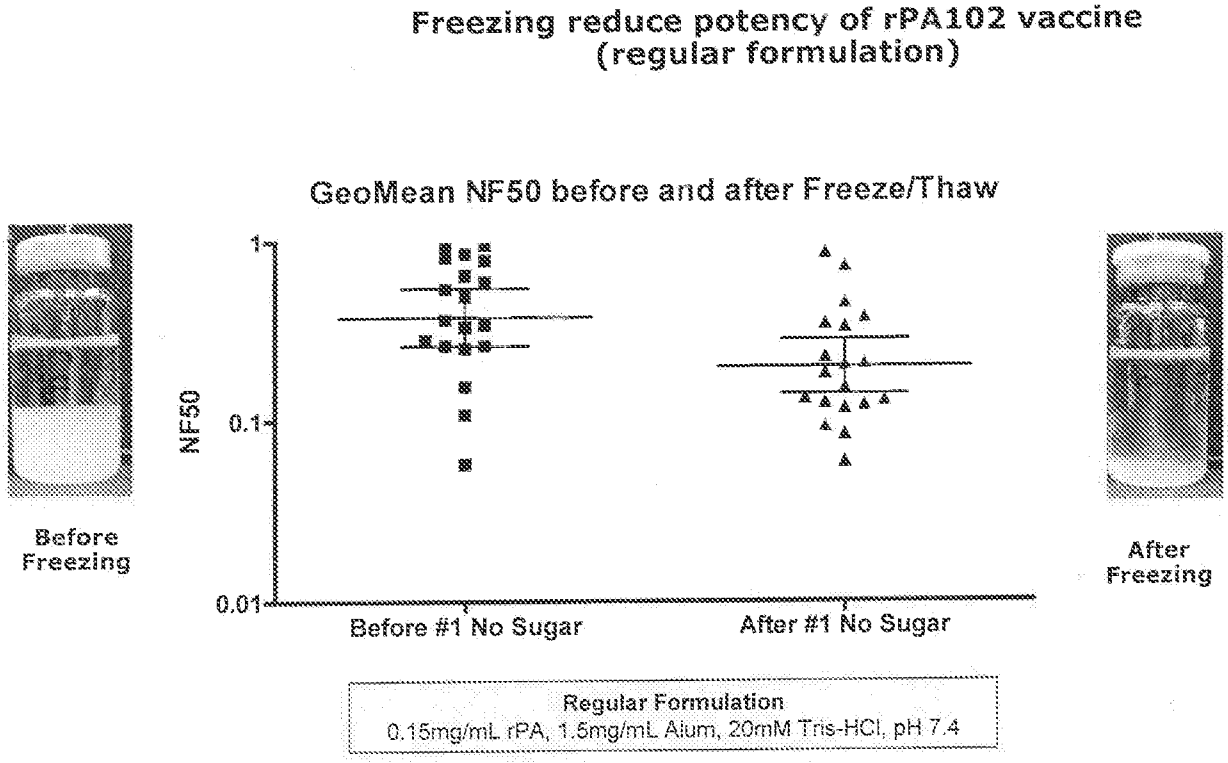

Freezing reduce potency of rPA102 vaccine
(regular formulation)
GeoMean NF50 before and after Freeze/Thaw
NF50
1
0.1
0.01
Before #1 No Sugar
After #1 No Sugar
Before Freezing
After Freezing
Regular Formulation
0.15mg/mL rPA, 1.5mg/mL Alum, 20mM Tris-HCl, pH 7.4
• After freezing, geomean NF50 of 19 mice is statistically lower than that before freezing
• Note: Potency drop correlates with gel collapse

FIG. 4

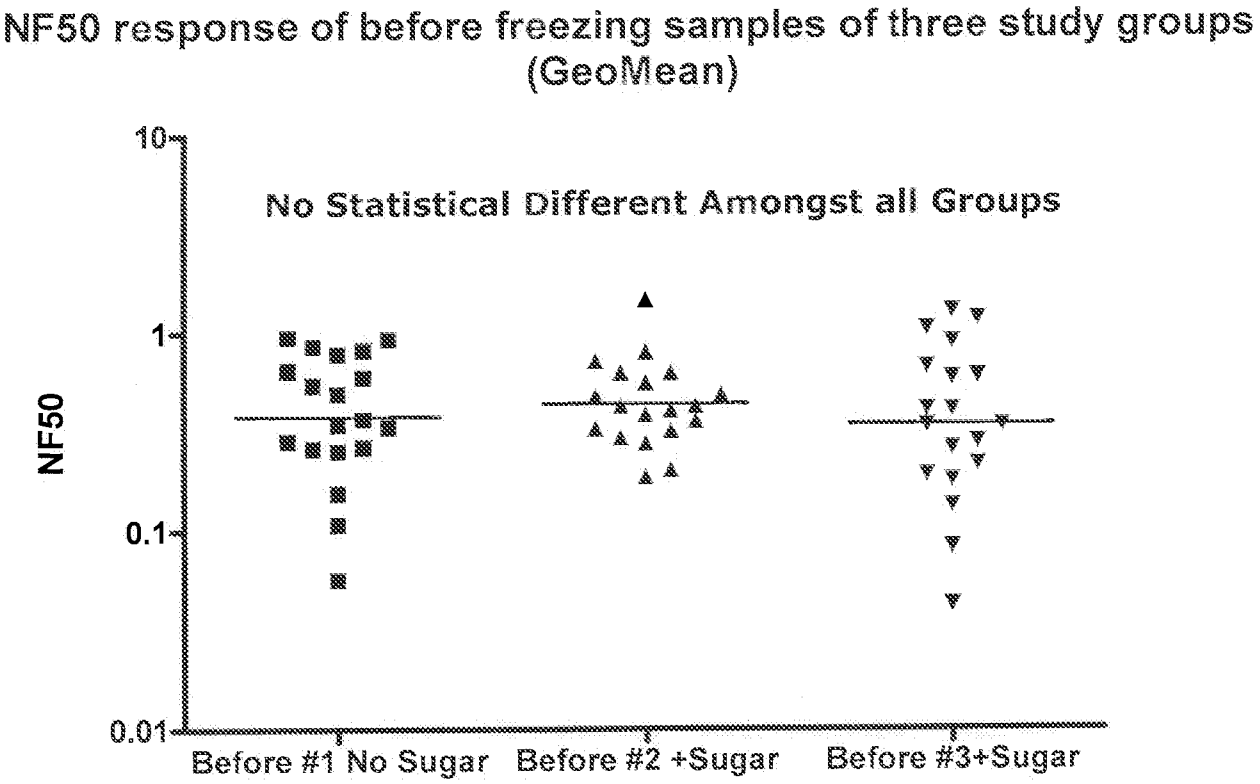

NF50 response of before freezing samples of three study groups
(GeoMean)
No Statistical Different Amongst all Groups
NF50
10
1
0.1
0.01
Before #1 No Sugar
Before #2 +Sugar
Before #3+Sugar

FIG. 5

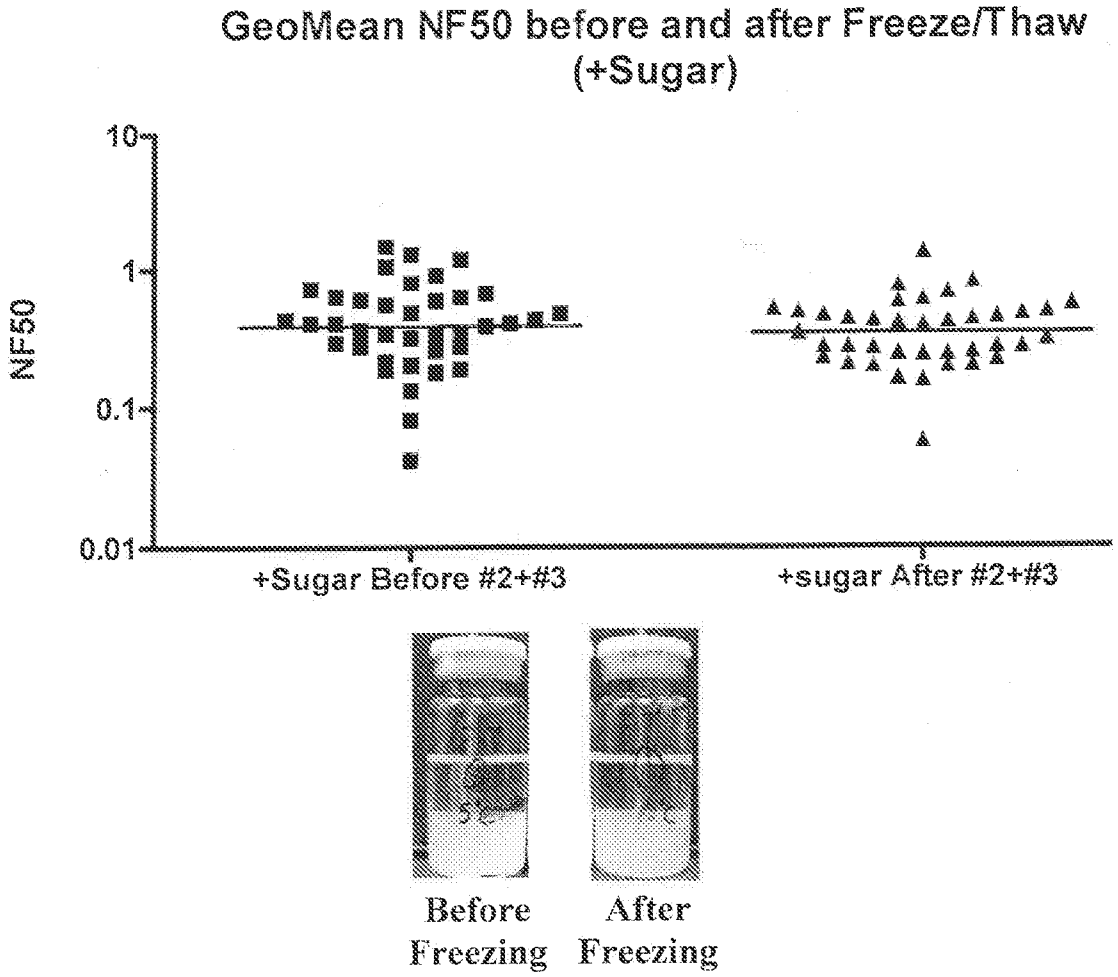

GeoMean NF50 before and after Freeze/Thaw
(+Sugar)
NF50
10
1
0.1
0.01
+Sugar Before #2+#3
+sugar After #2+#3
Before
Freezing
After
Freezing

FIG. 6

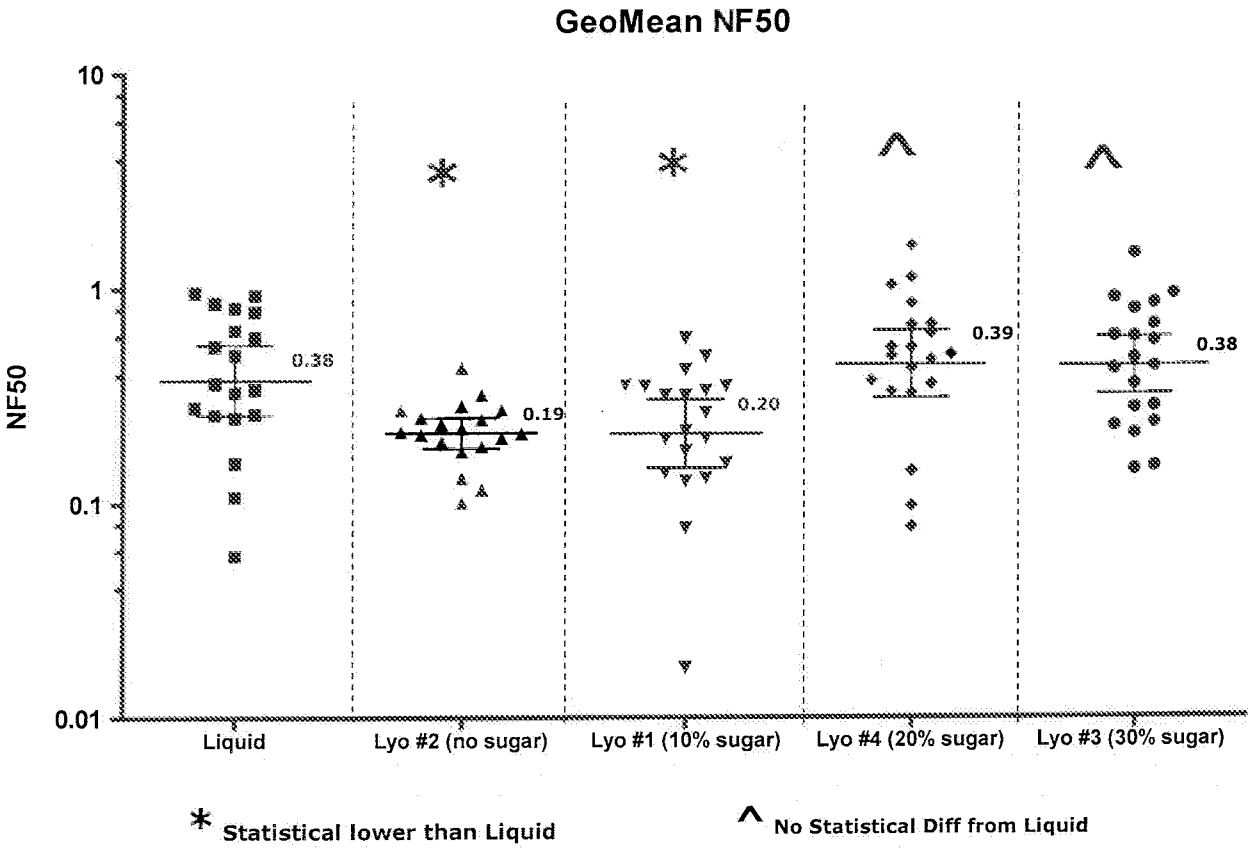

GeoMean NF50
NF50
10
1
0.1
0.01
0.38
0.19
0.20
0.39
0.38
Liquid
Lyo #2 (no sugar)
Lyo #1 (10% sugar)
Lyo #4 (20% sugar)
Lyo #3 (30% sugar)
* Statistical lower than Liquid
^ No Statistical Diff from Liquid

FIG. 7

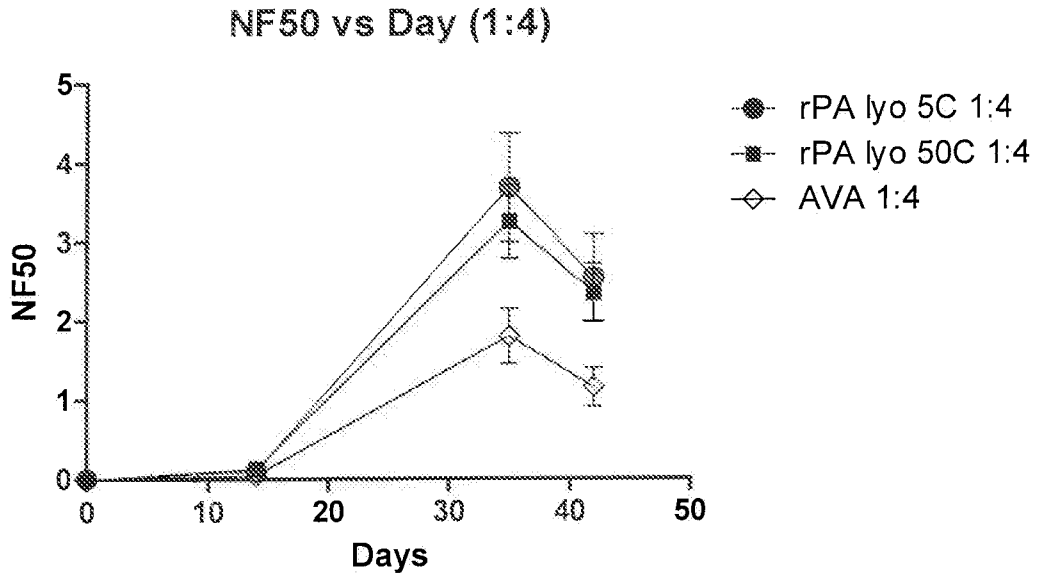

NF50 vs Day (1:4)
NF50
Days
0
1
2
3
4
5
0
10
20
30
40
50
rPA lyo 5C 1:4
rPA lyo 50C 1:4
AVA 1:4

FIG. 8A

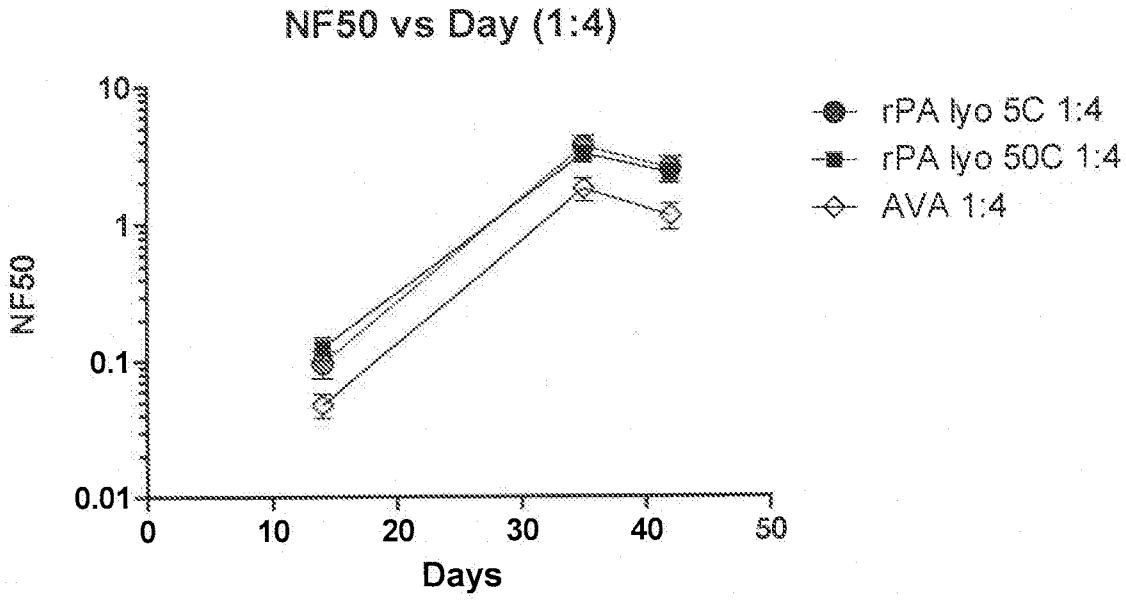

NF50 vs Day (1:4)
NF50
Days
10
1
0.1
0.01
0
10
20
30
40
50
rPA lyo 5C 1:4
rPA lyo 50C 1:4
AVA 1:4

FIG. 8B

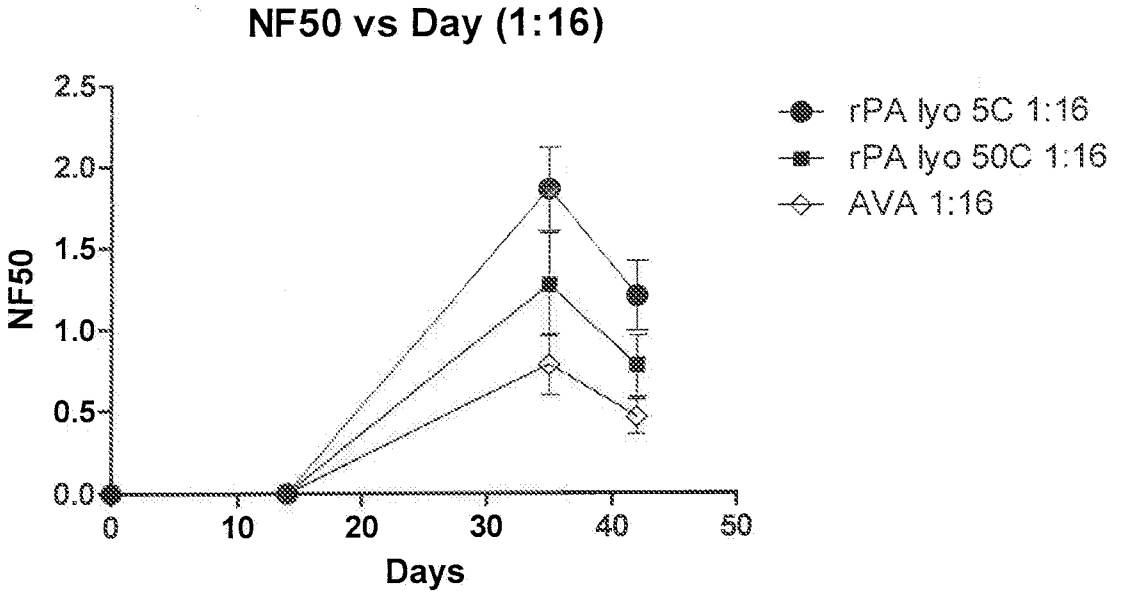

NF50 vs Day (1:16)
NF50
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Days
rPA lyo 5C 1:16
rPA lyo 50C 1:16
AVA 1:16

FIG. 9A

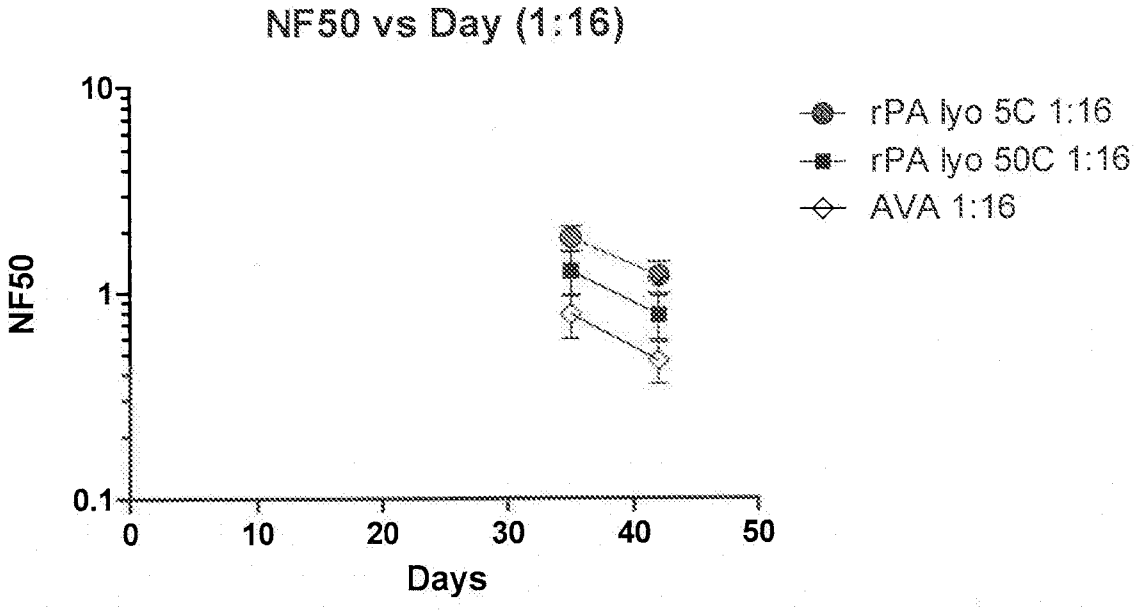

NF50 vs Day (1:16)
NF50
10
1
0.1
0
10
20
30
40
50
Days
rPA lyo 5C 1:16
rPA lyo 50C 1:16
AVA 1:16

FIG. 9B

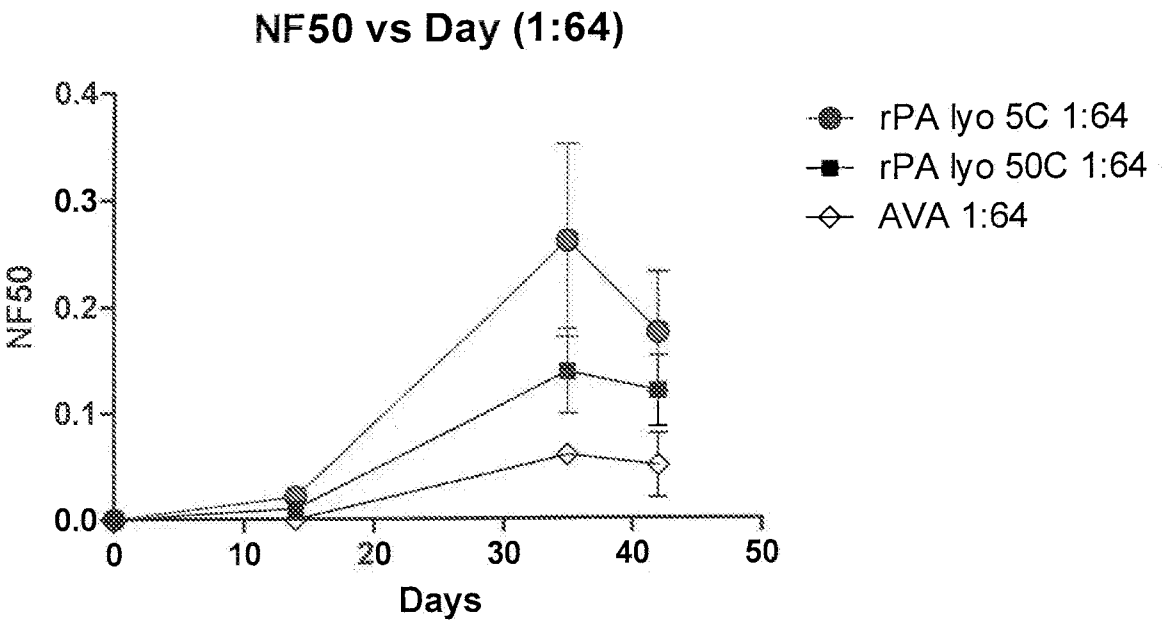


FIG. 10A

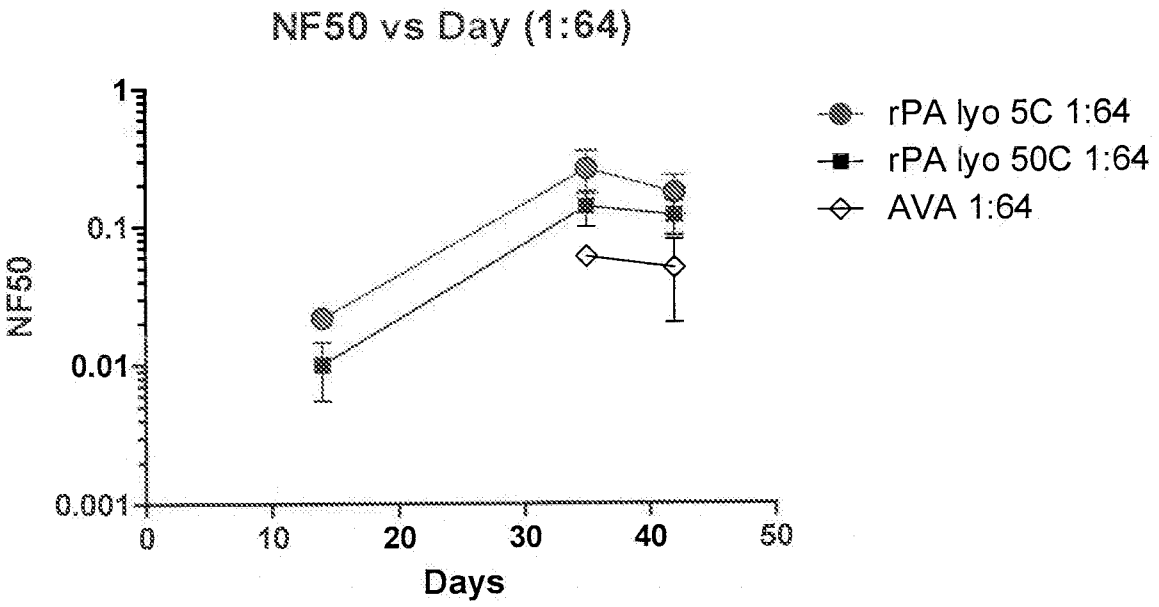


FIG. 10B

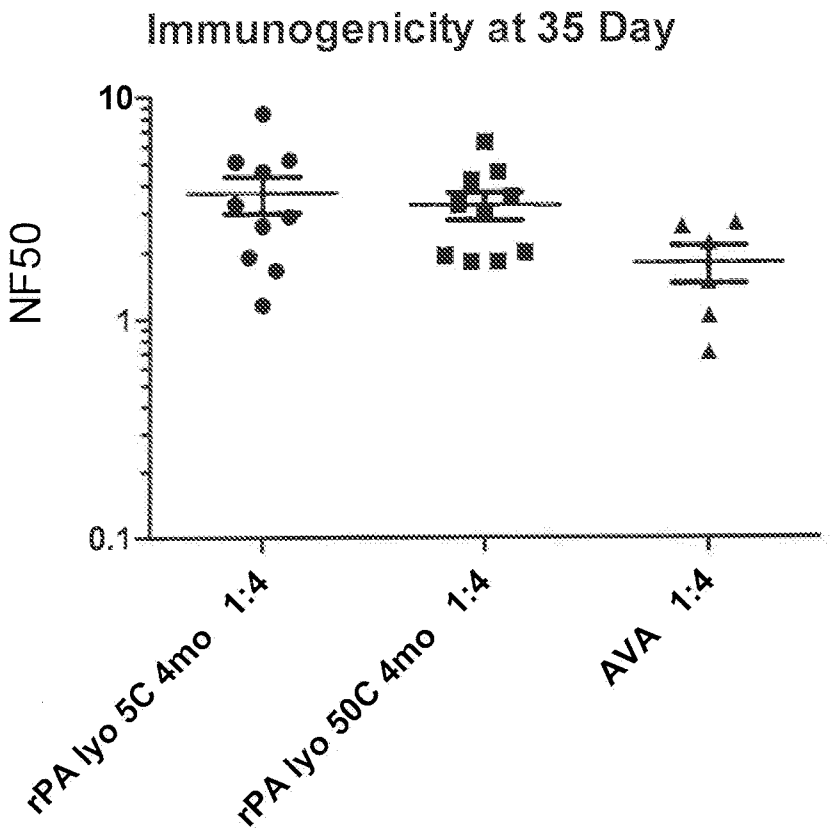


FIG. 11A

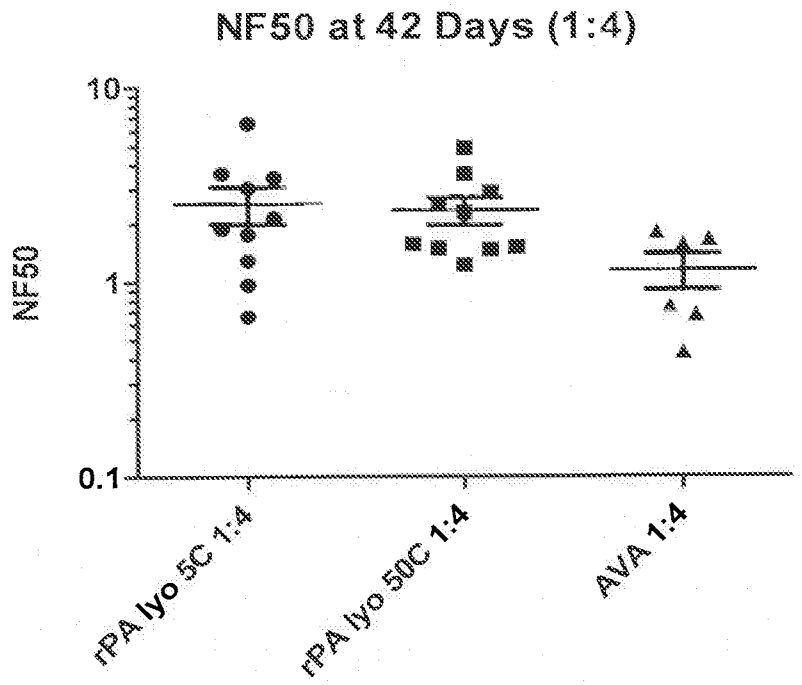


FIG. 11B

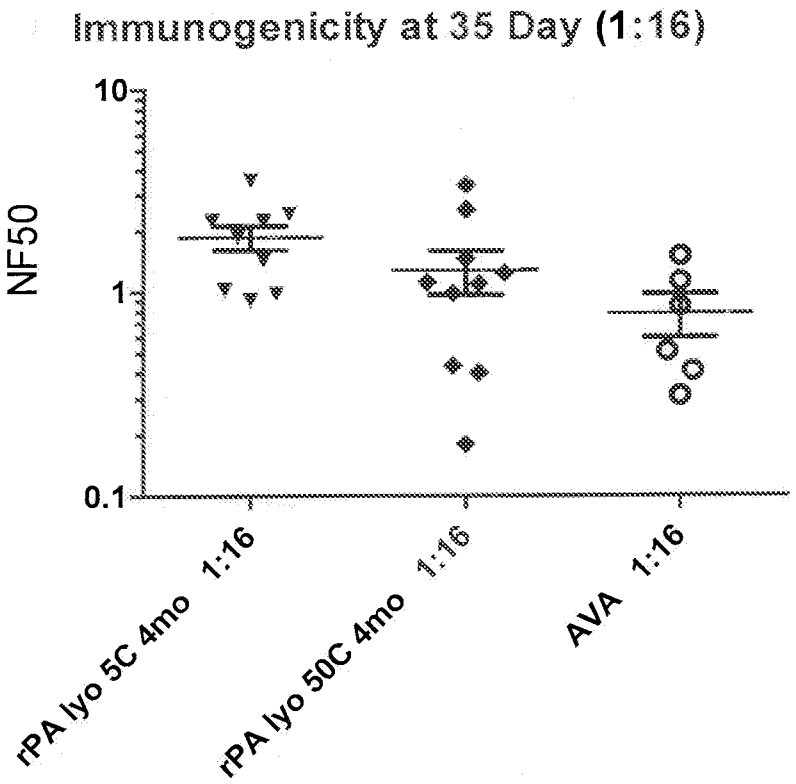


FIG. 12A

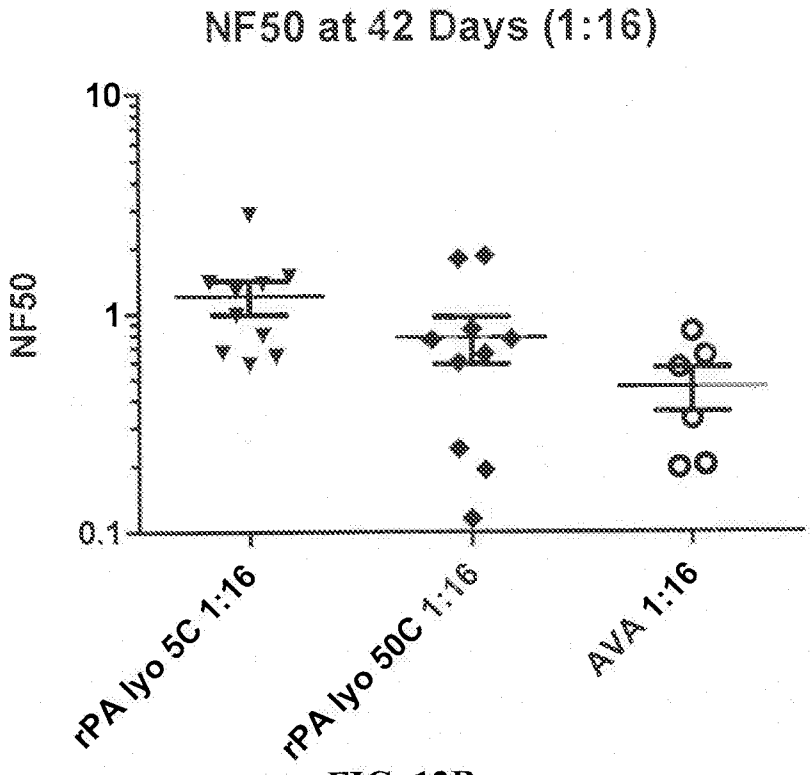


FIG. 12B

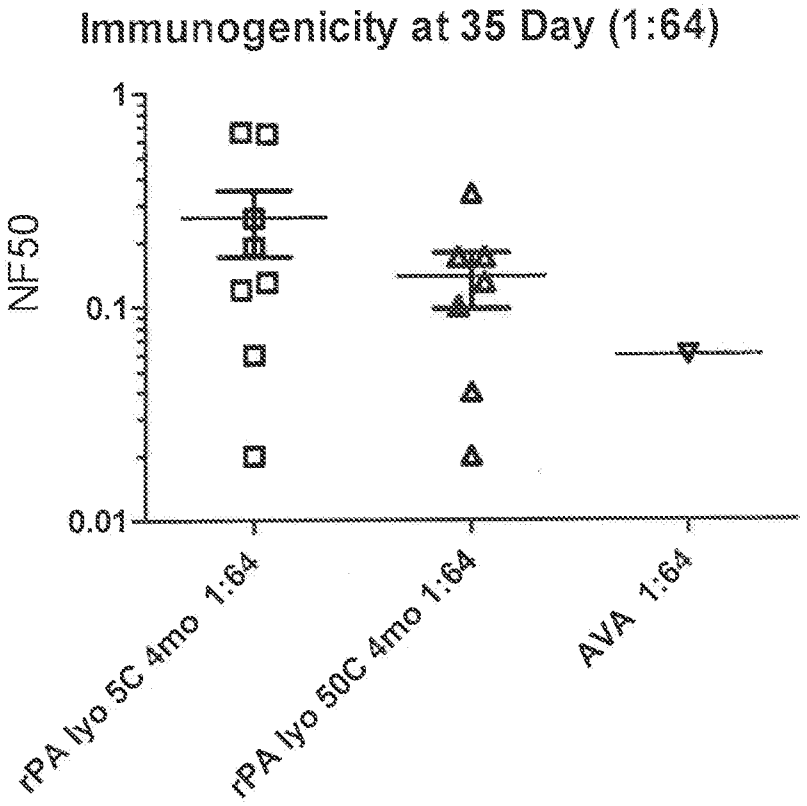


FIG. 13A

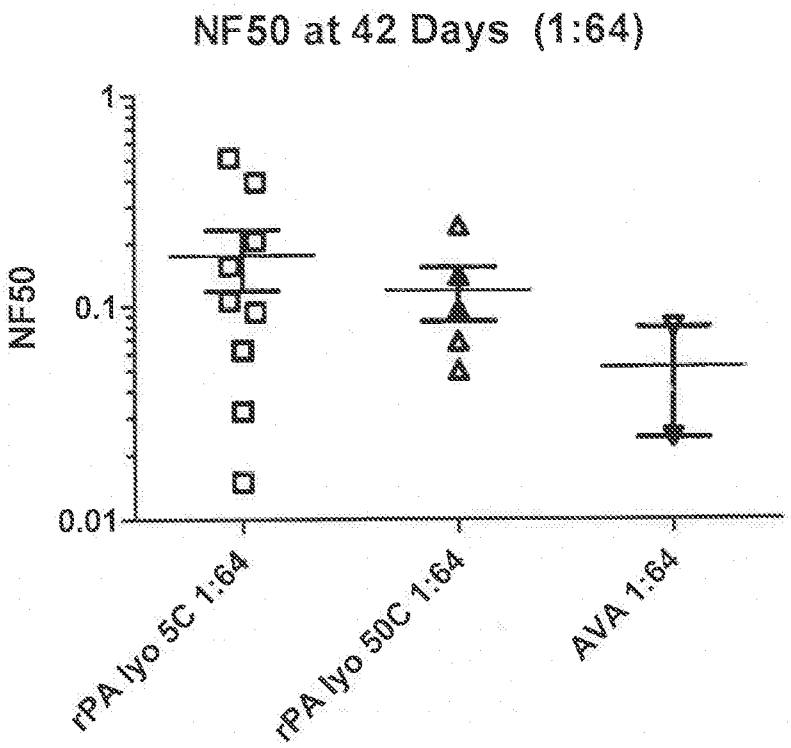


FIG. 13B

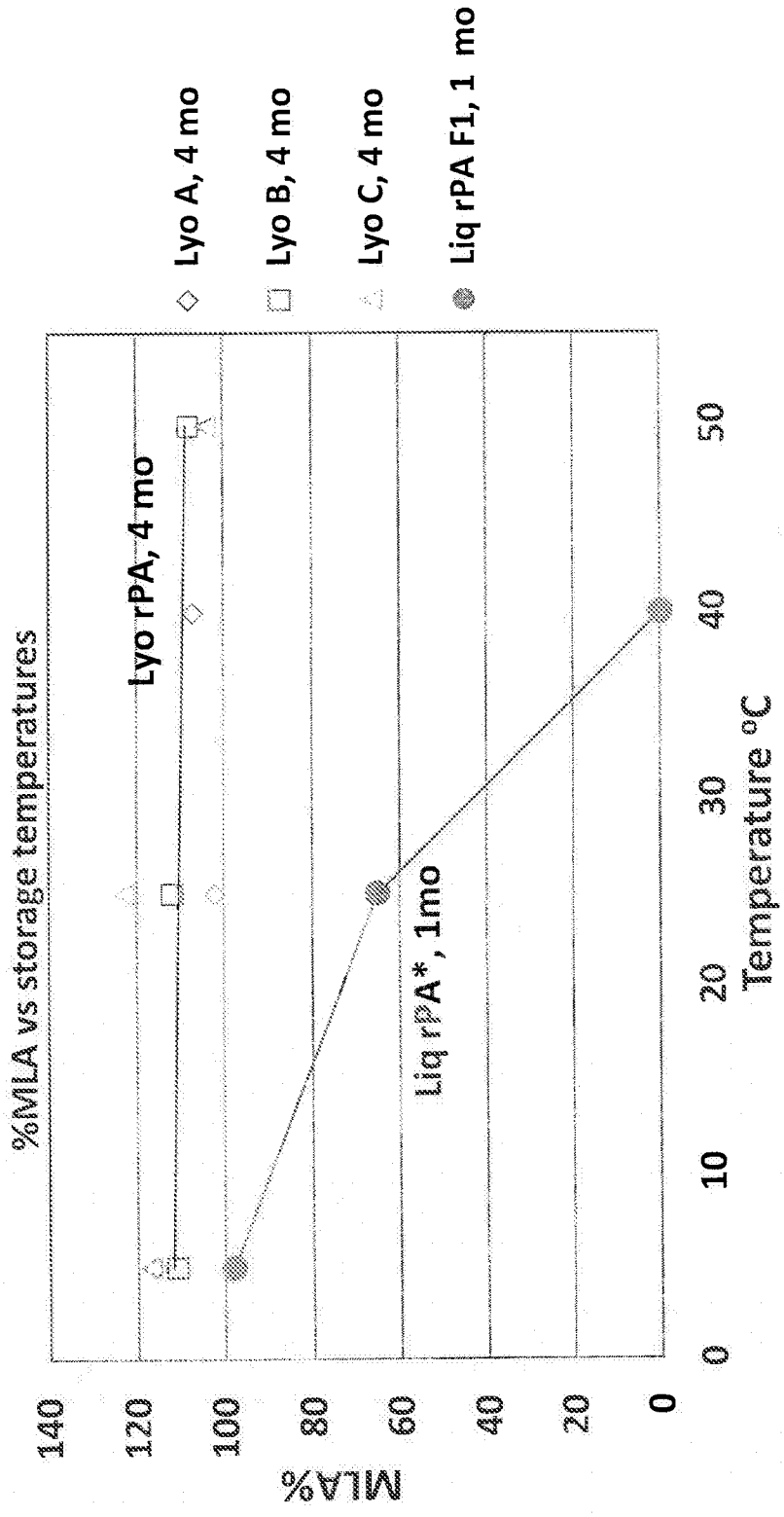

%MLA vs storage temperatures
Lyo A, 4 mo
Lyo B, 4 mo
Lyo C, 4 mo
Liq rPA F1, 1 mo
Lyo rPA, 4 mo
Liq rPA*, 1mo
MLA%
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Temperature °C

FIG. 14

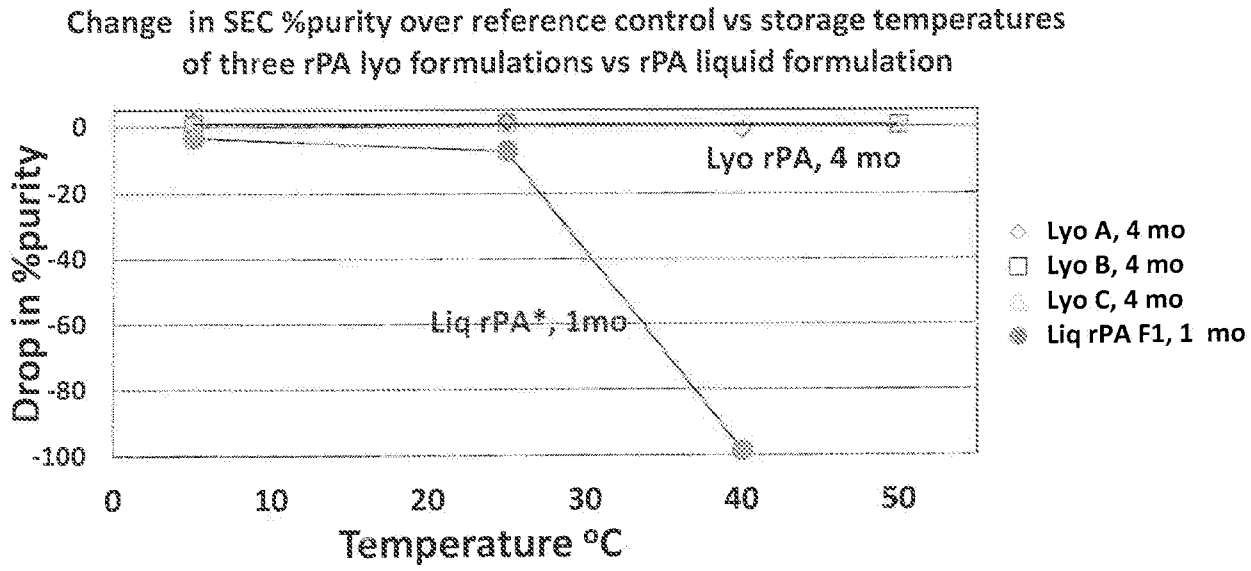


FIG. 15A

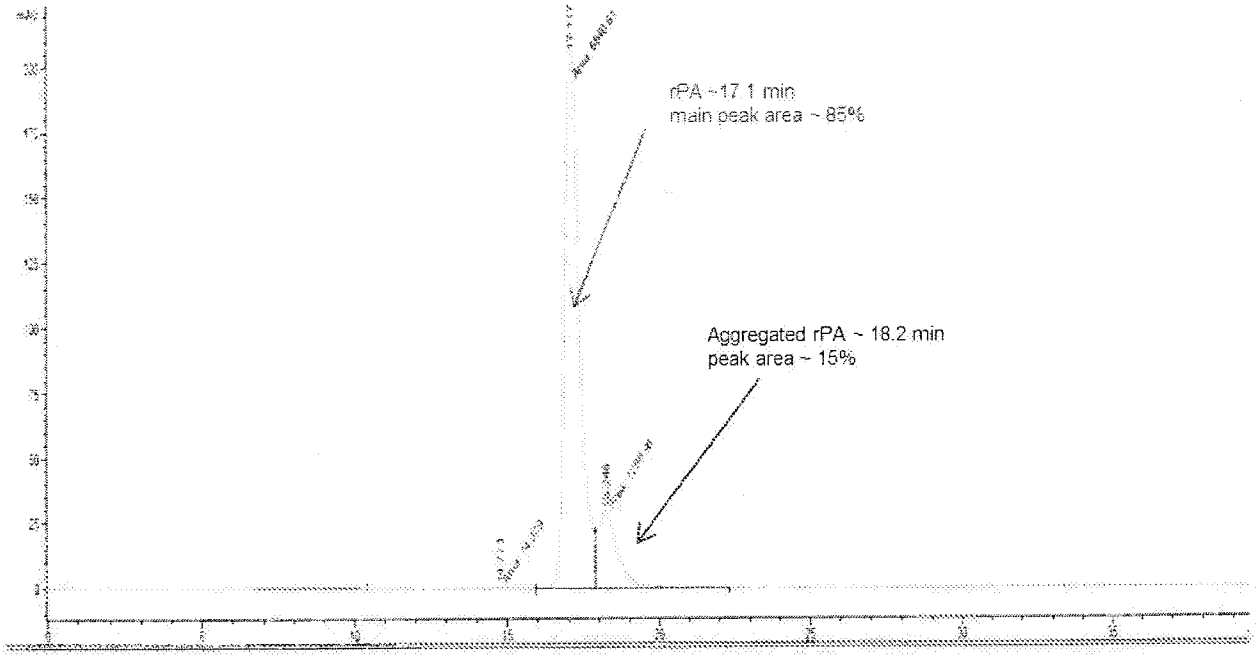


FIG. 15B

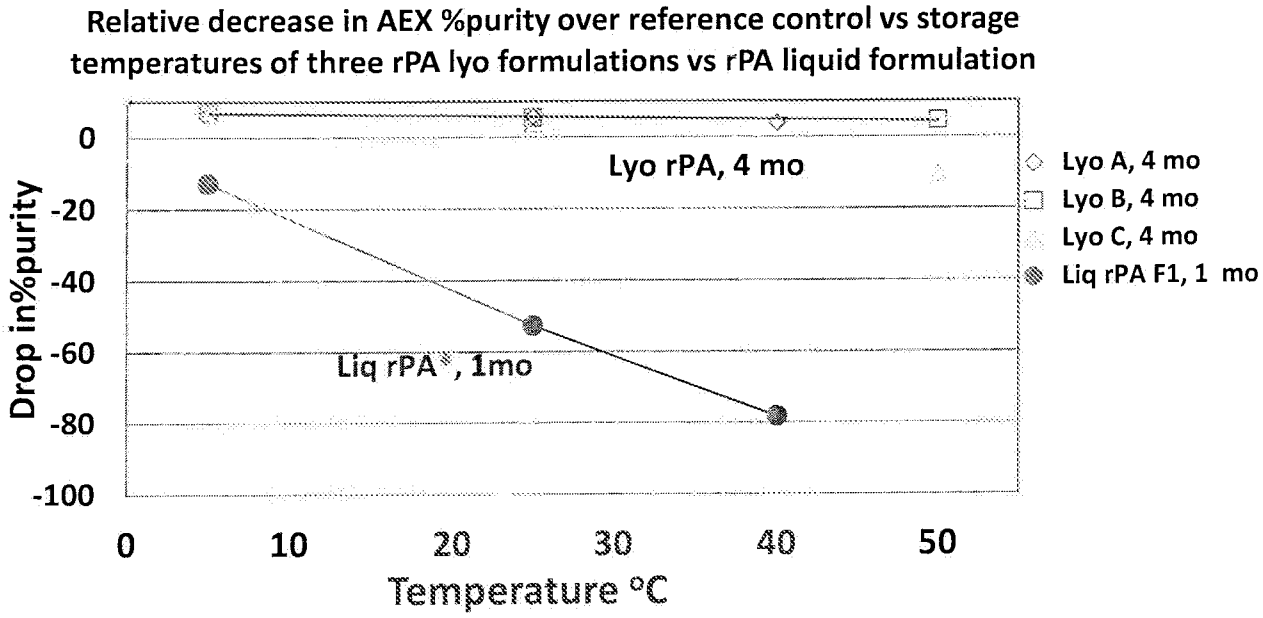


FIG. 16A

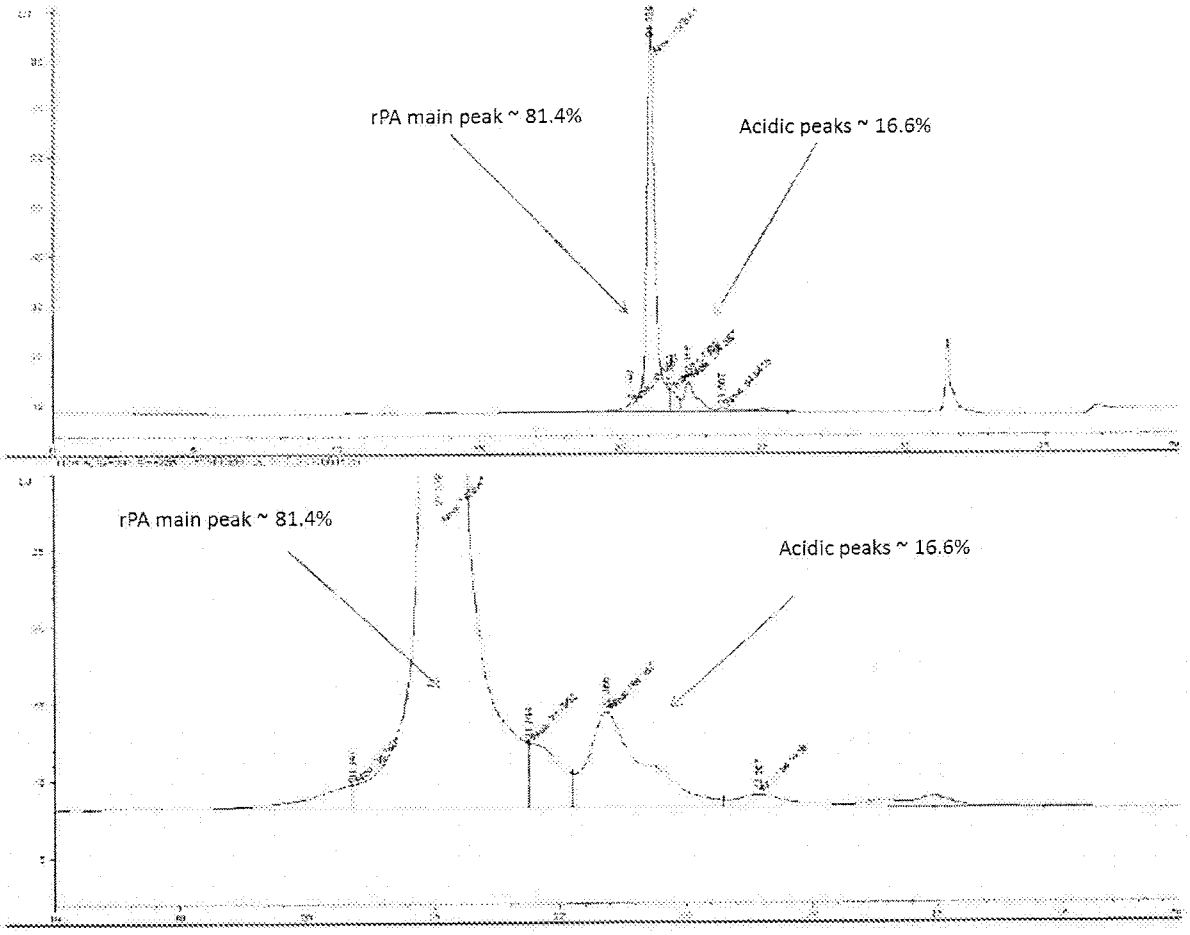


FIG. 16B

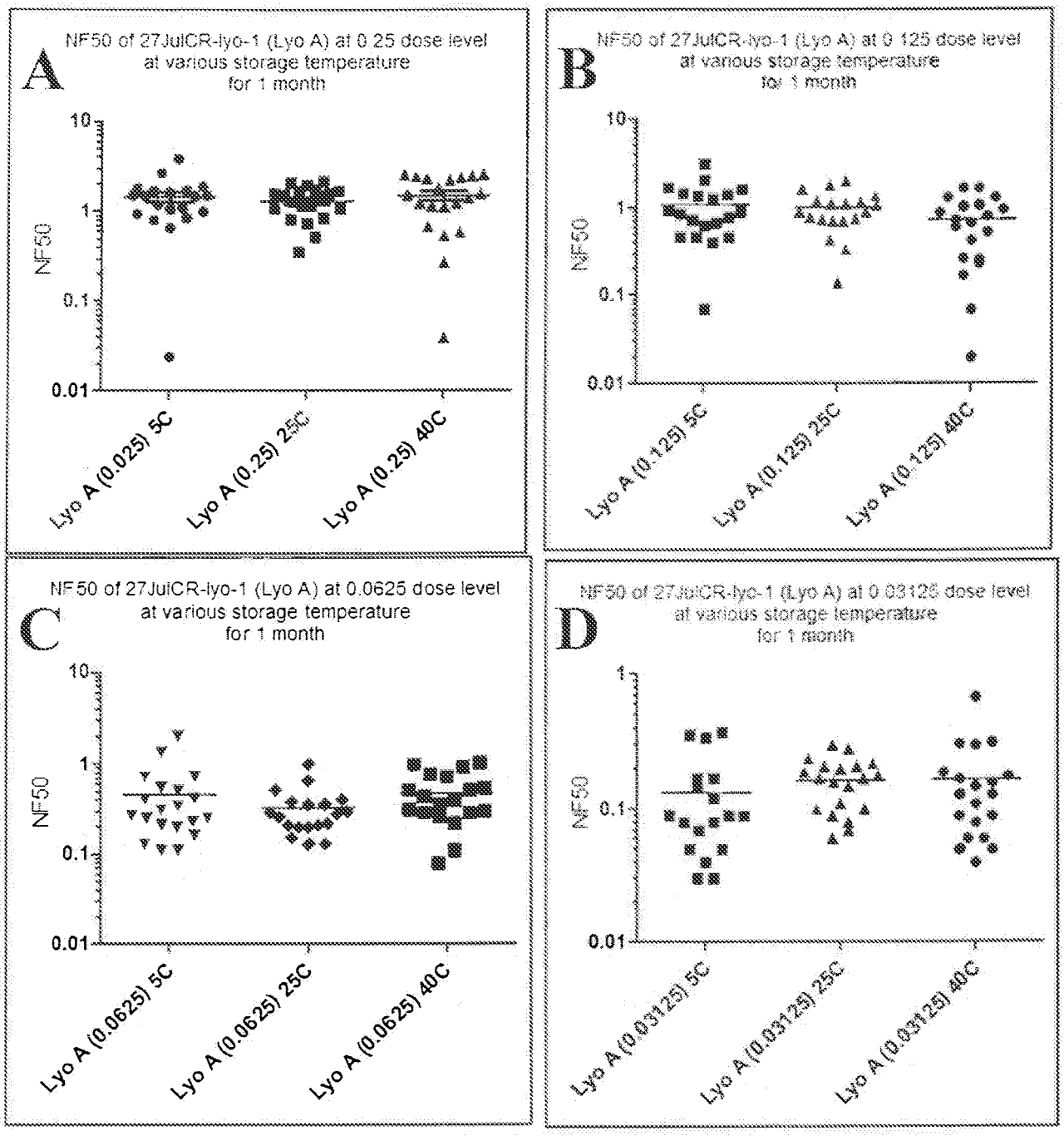

A
NF50 of 27JulCR-lyo-1 (Lyo A) at 0.25 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo A (0.025) 5C
Lyo A (0.25) 25C
Lyo A (0.25) 40C
B
NF50 of 27JulCR-lyo-1 (Lyo A) at 0.125 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo A (0.125) 5C
Lyo A (0.125) 25C
Lyo A (0.125) 40C
C
NF50 of 27JulCR-lyo-1 (Lyo A) at 0.0625 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo A (0.0625) 5C
Lyo A (0.0625) 25C
Lyo A (0.0625) 40C
D
NF50 of 27JulCR-lyo-1 (Lyo A) at 0.03125 dose level
at various storage temperature
for 1 month
NF50
1
0.1
0.01
Lyo A (0.03125) 5C
Lyo A (0.03125) 25C
Lyo A (0.03125) 40C

FIG. 17A-D

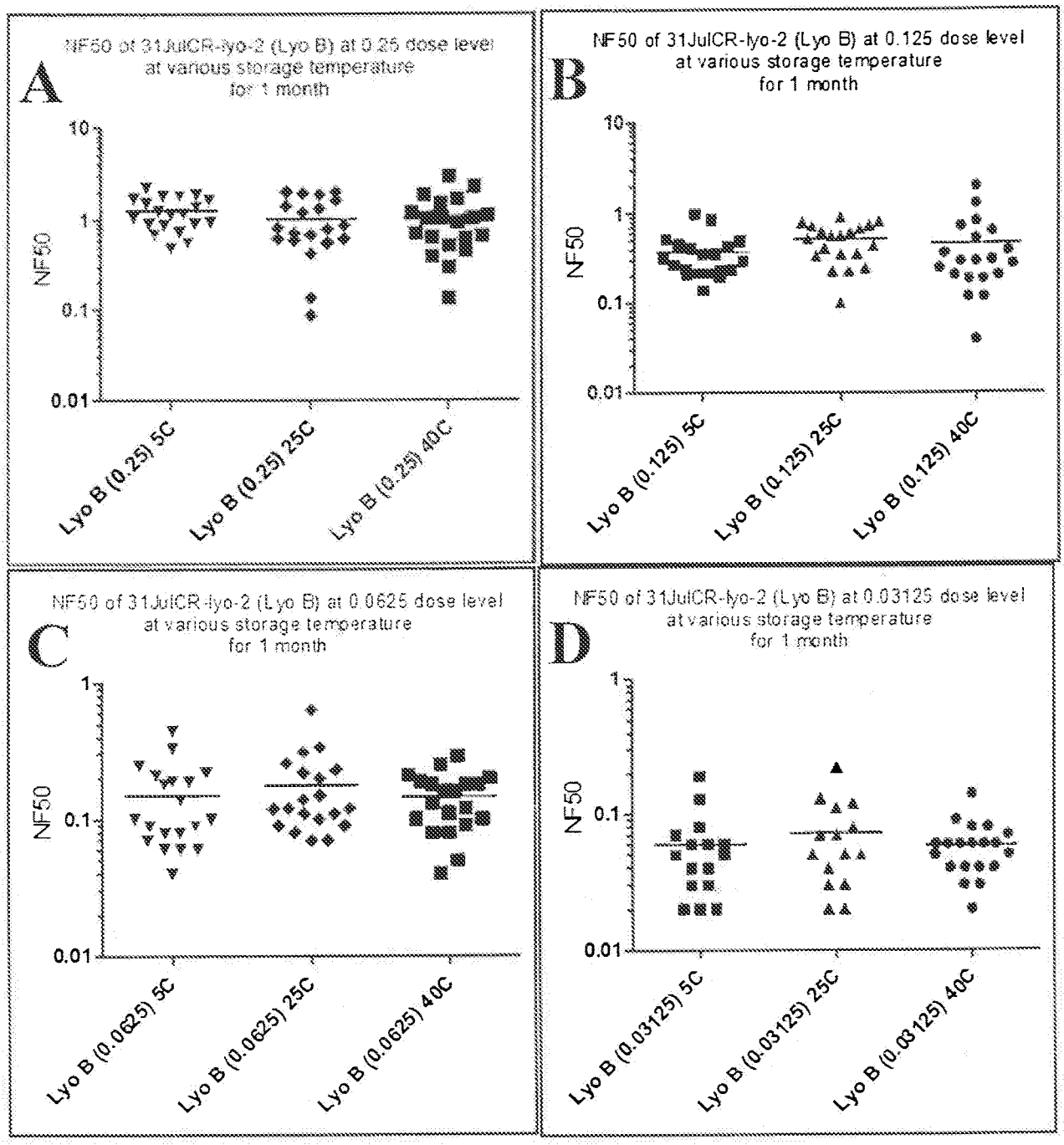

A
NF50 of 31JulCR-lyo-2 (Lyo B) at 0.25 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo B (0.25) 5C
Lyo B (0.25) 25C
Lyo B (0.25) 40C
B
NF50 of 31JulCR-lyo-2 (Lyo B) at 0.125 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo B (0.125) 5C
Lyo B (0.125) 25C
Lyo B (0.125) 40C
C
NF50 of 31JulCR-lyo-2 (Lyo B) at 0.0625 dose level
at various storage temperature
for 1 month
NF50
1
0.1
0.01
Lyo B (0.0625) 5C
Lyo B (0.0625) 25C
Lyo B (0.0625) 40C
D
NF50 of 31JulCR-lyo-2 (Lyo B) at 0.03125 dose level
at various storage temperature
for 1 month
NF50
1
0.1
0.01
Lyo B (0.03125) 5C
Lyo B (0.03125) 25C
Lyo B (0.03125) 40C

FIG. 18A-D

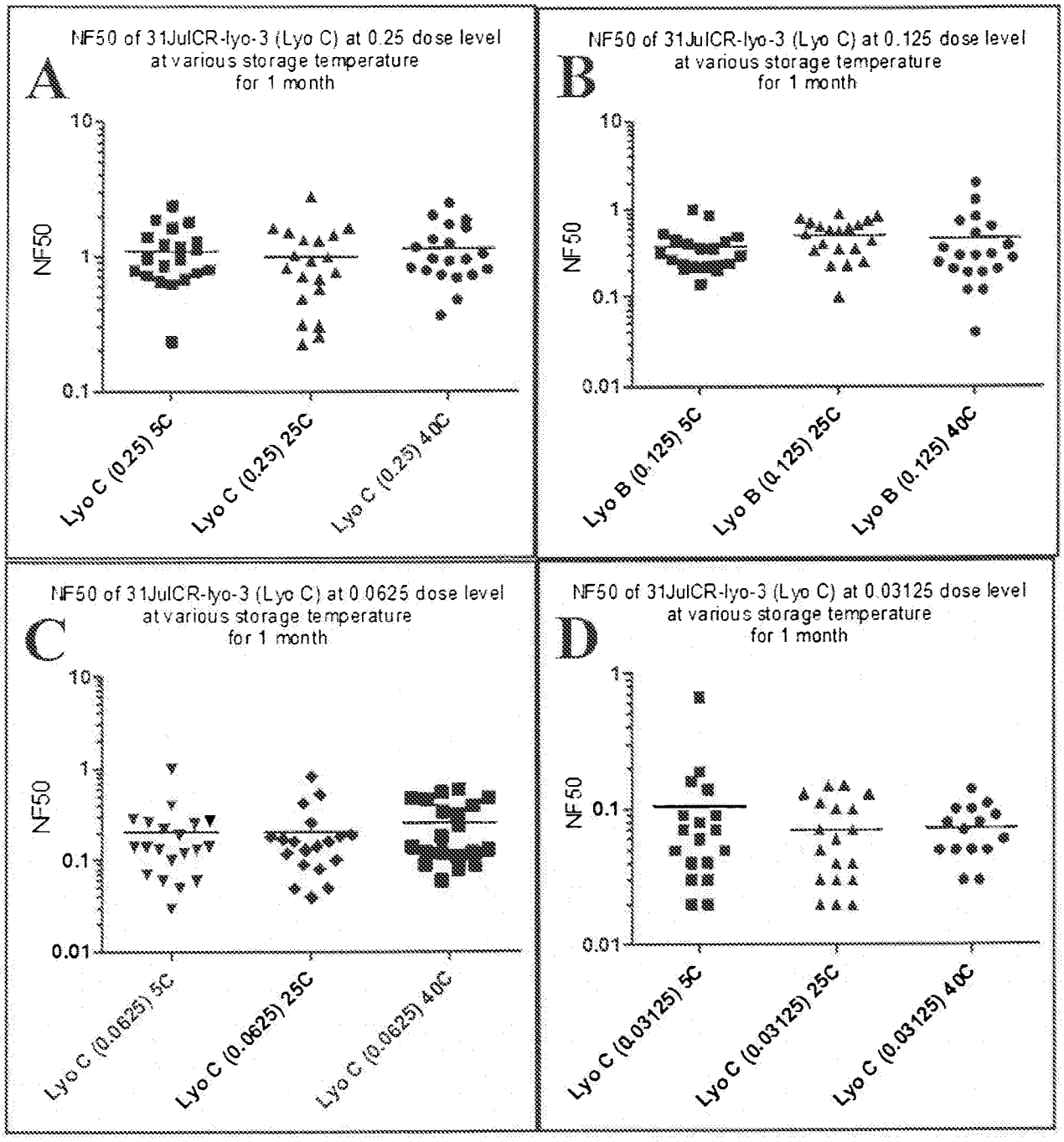

A
NF50 of 31JulCR-lyo-3 (Lyo C) at 0.25 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
Lyo C (0.25) 5C
Lyo C (0.25) 25C
Lyo C (0.25) 40C
B
NF50 of 31JulCR-lyo-3 (Lyo C) at 0.125 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo B (0.125) 5C
Lyo B (0.125) 25C
Lyo B (0.125) 40C
C
NF50 of 31JulCR-lyo-3 (Lyo C) at 0.0625 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
Lyo C (0.0625) 5C
Lyo C (0.0625) 25C
Lyo C (0.0625) 40C
D
NF50 of 31JulCR-lyo-3 (Lyo C) at 0.03125 dose level
at various storage temperature
for 1 month
NF50
1
0.1
0.01
Lyo C (0.03125) 5C
Lyo C (0.03125) 25C
Lyo C (0.03125) 40C

FIG. 19A-D

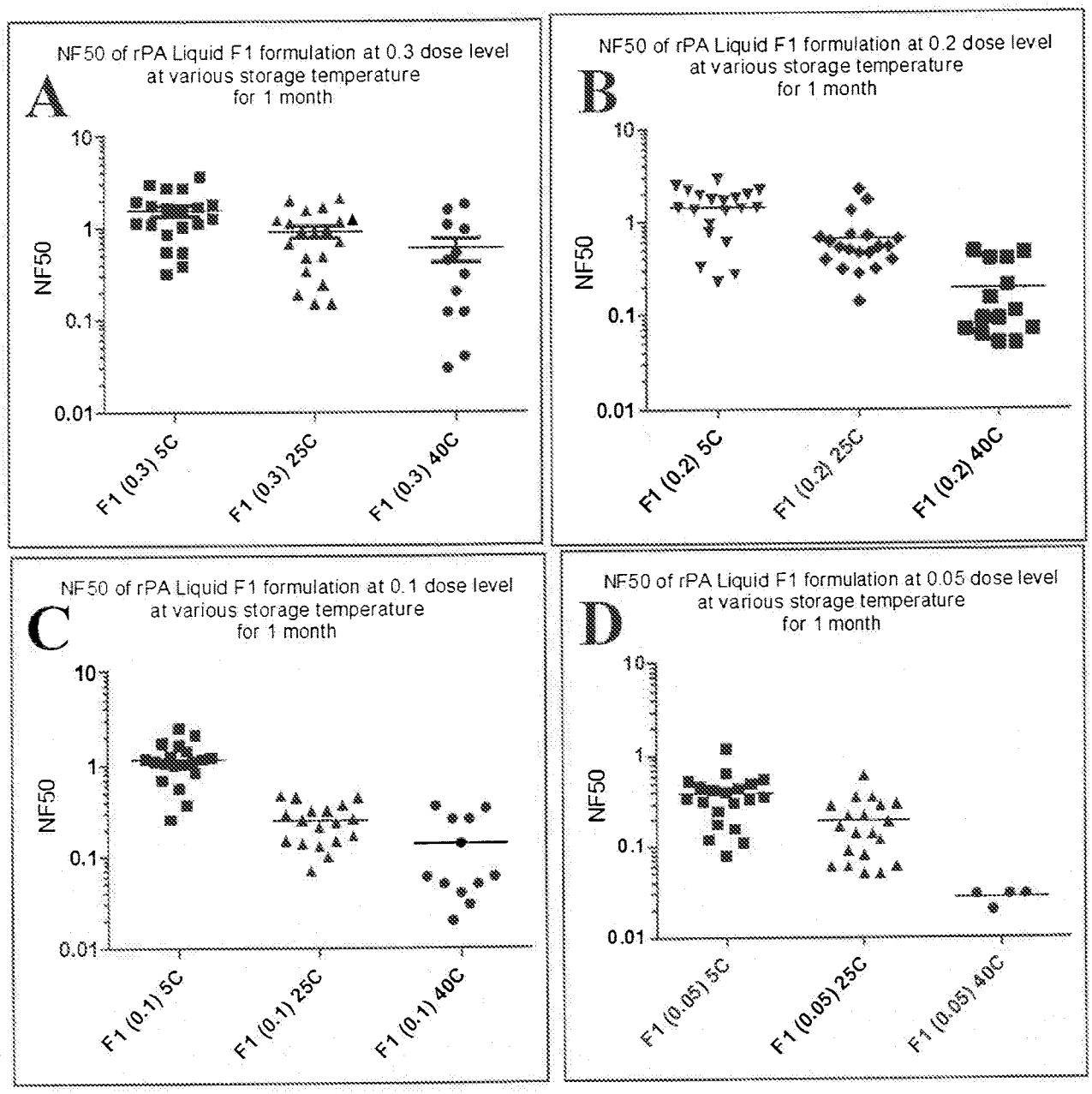

A
NF50 of rPA Liquid F1 formulation at 0.3 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
F1 (0.3) 5C
F1 (0.3) 25C
F1 (0.3) 40C
B
NF50 of rPA Liquid F1 formulation at 0.2 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
F1 (0.2) 5C
F1 (0.2) 25C
F1 (0.2) 40C
C
NF50 of rPA Liquid F1 formulation at 0.1 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
F1 (0.1) 5C
F1 (0.1) 25C
F1 (0.1) 40C
D
NF50 of rPA Liquid F1 formulation at 0.05 dose level
at various storage temperature
for 1 month
NF50
10
1
0.1
0.01
F1 (0.05) 5C
F1 (0.05) 25C
F1 (0.05) 40C

FIG. 20A-D

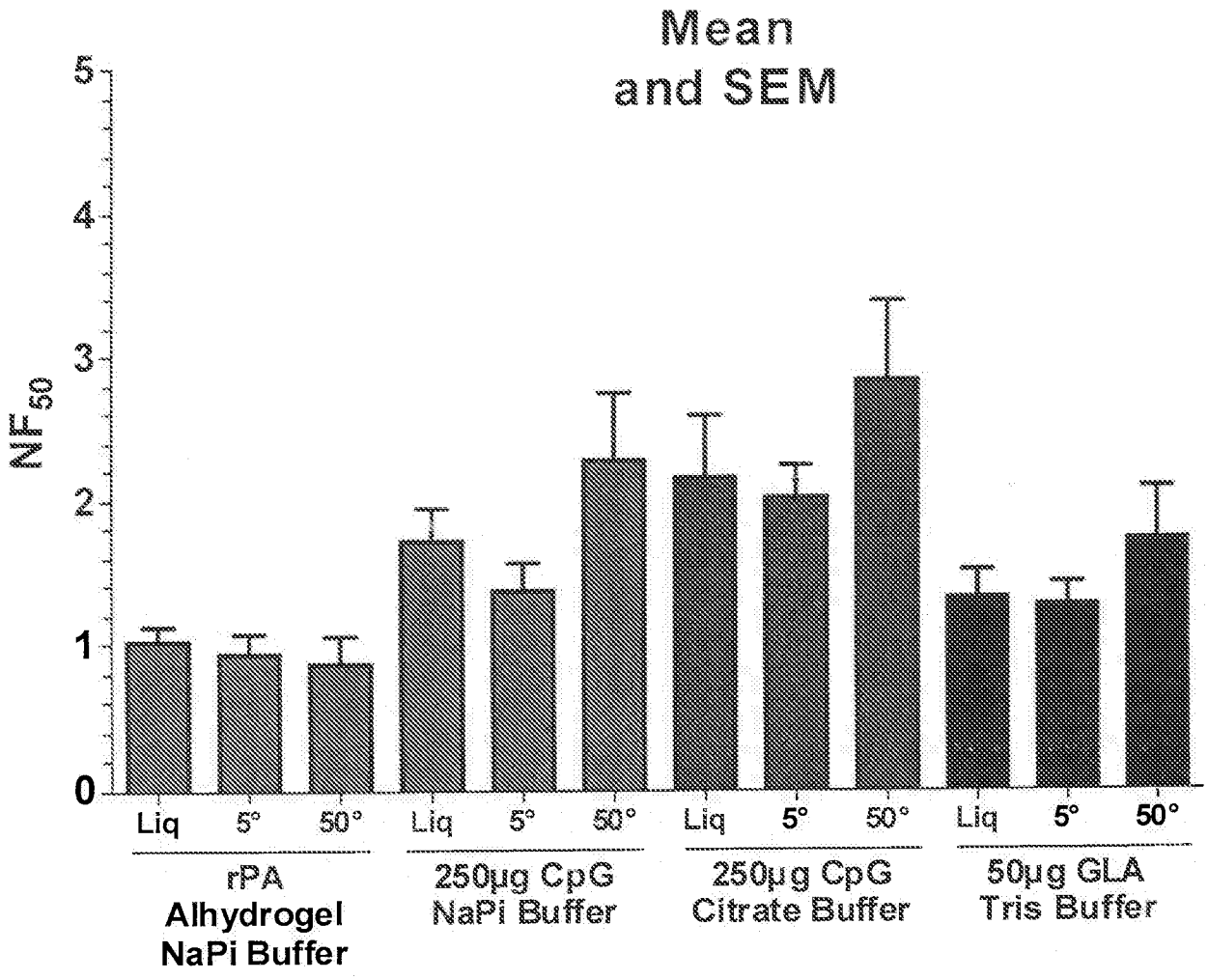

Mean
and SEM
NF50
5
4
3
2
1
0
Liq
5°
50°
Liq
5°
50°
Liq
5°
50°
Liq
5°
50°
rPA
Alhydrogel
NaPi Buffer
250µg CpG
NaPi Buffer
250µg CpG
Citrate Buffer
50µg GLA
Tris Buffer

FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/047712

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/18 (2013.01)
USPC - 424/499
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 9/00, 9/18, 39/02, 39/385, 39/39, 39/40 (2013.01)
USPC - 424/400, 489, 499, 184.1, 193.1, 197.11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 9/00, 9/18, 39/02, 39/385, 39/39, 39/40 (2013.01)

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/084298 A1 (WATKINSON et al) 29 July 2010 (29.07.2010) entire document	1, 4-6, 8, 9
X	US 2010/0158951 A1 (RANDOLPH et al) 24 June 2010 (24.06.2010) entire document	2, 3, 47-49
A	US 2011/0229507 A1 (KAISHEVA) 22 September 2011 (22.09.2011) entire document	1-6, 8, 9, 47-49
A	WANG et al., "Selection of Adjuvants for Enhanced Vaccine Potency," World Journal of Vaccines, Vol. 1, Pgs. 33-78, 12 October 2011 (12.10.2011) entire document	1-6, 8, 9, 47-49

☐ Further documents are listed in the continuation of Box C ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 November 2013	26 NOV 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/047712

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7, 10-46, 50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

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(43)申请公布日 2015.03.25

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PCT/US2013/047712 2013.06.25
(87)PCT国际申请的公布数据
WO2014/004578 EN 2014.01.03
(71)申请人 新兴产品开发盖瑟斯堡有限公司
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代理人 孟锐
(51)Int.Cl.
A61K 9/18(2006.01)

权利要求书3页 说明书36页
序列表10页 附图19页

(54)发明名称

温度稳定性疫苗制剂

(57)摘要

本发明提供在经历冷冻和解冻条件之后保持稳定的疫苗抗原，如炭疽保护性抗原的制剂。还提供使用所述制剂来制备疫苗的方法。包含所述制剂的疫苗适用于例如预防、抑制或减轻如与炭疽感染有关的疾病或感染。

CN 104470506 A

1. 一种用于制备冻干疫苗的组合物，其包含至少一种吸附于铝佐剂的抗原和至少20％ (w/v) 的非还原糖。

2. 一种温度稳定性液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原和至少20％ (w/v) 的糖。

3. 一种组合物，其在冻干之前包含至少一种吸附于铝佐剂的抗原和至少20％ (w/v) 的非还原糖，其中在复原之后，所述非还原糖是至少6％ (w/v)。

4. 如权利要求1至3中任一项所述的组合物，其中所述组合物包含表面活性剂。

5. 一种用于制备冻干疫苗的组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂和至少15％ (w/v) 的糖。

6. 一种温度稳定性液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂和至少15％ (w/v) 的糖。

7. 如权利要求1至6中任一项所述的组合物，其中所述组合物进一步包含氨基酸。

8. 一种用于制备冻干疫苗的组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂、氨基酸和至少10％ (w/v) 的糖。

9. 一种稳定的液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂、氨基酸和至少10％ (w/v) 的糖。

10. 如权利要求1至9中任一项所述的组合物，其中所述铝佐剂选自磷酸铝和硫酸铝。

11. 如权利要求1至9中任一项所述的组合物，其中所述铝佐剂是氢氧化铝。

12. 如权利要求11所述的组合物，其中所述组合物含有约0.5至1.5mg/ml氢氧化铝。

13. 如权利要求12所述的组合物，其中所述组合物含有约0.5mg/ml氢氧化铝。

14. 如权利要求12所述的组合物，其中所述组合物含有约1.5mg/ml氢氧化铝。

15. 如权利要求4至14中任一项所述的组合物，其中所述表面活性剂选自聚山梨醇酯80和聚山梨醇酯20。

16. 如权利要求15所述的组合物，其中所述表面活性剂是聚山梨醇酯80。

17. 如权利要求4至16中任一项所述的组合物，其中所述组合物含有约0.020％或0.025％ (w/v) 的表面活性剂。

18. 如权利要求1至17所述的组合物，其中所述糖是选自海藻糖、蔗糖和其组合的非还原糖。

19. 如权利要求18所述的组合物，其中所述糖是海藻糖。

20. 如权利要求18所述的组合物，其中所述糖是蔗糖。

21. 如权利要求18所述的组合物，其中所述糖是海藻糖和蔗糖。

22. 如权利要求5至21中任一项所述的组合物，其中所述组合物含有约15-40％ (w/v) 的糖。

23. 如权利要求1至22中任一项所述的组合物，其中所述组合物含有约20-40％ (w/v) 的糖。

24. 如权利要求23所述的组合物，其中所述组合物含有约20-35％ (w/v) 的糖。

25. 如权利要求24所述的组合物，其中所述组合物含有约25-40％ (w/v) 的糖。

26. 如权利要求1至25中任一项所述的组合物，其中所述组合物含有大于约20％、21％、22％、23％、24％和25％ (w/v) 的糖。

27. 如权利要求 1 至 26 中任一项所述的组合物，其中所述抗原是炭疽抗原。

28. 如权利要求 27 所述的组合物，其中所述炭疽抗原是保护性抗原。

29. 如权利要求 28 所述的组合物，其中所述保护性抗原与 SEQ ID NO :2 的多肽具有至少约 80％的同一性。

30. 如权利要求 28 至 29 中任一项所述的组合物，其中所述组合物包含约 150-500 μ g/ml 保护性抗原。

31. 如权利要求 30 所述的组合物，其中所述组合物包含约 150、175、200、225、250、275、300、325、400、375、400、425、450、475 或 500 μ g/ml 保护性抗原

32. 如权利要求 31 所述的组合物，其中所述炭疽抗原是来自无毒力炭疽杆菌菌株的无细胞滤液。

33. 如权利要求 32 所述的组合物，其中所述无毒力炭疽杆菌菌株是 V770-NP1-R。

34. 如权利要求 7 至 33 中任一项所述的组合物，其中所述氨基酸选自精氨酸、丙氨酸、脯氨酸、甘氨酸和其任何组合。

35. 如权利要求 34 所述的组合物，其中所述组合物含有约 0.5-4％ (w/v) 的丙氨酸或精氨酸。

36. 如权利要求 40 所述的组合物，其中所述组合物含有约 2％ (w/v) 的丙氨酸或精氨酸。

37. 如权利要求 34 所述的组合物，其中所述组合物含有约 6-12％ (w/v) 的甘氨酸。

38. 如权利要求 37 所述的组合物，其中所述组合物含有约 10％ (w/v) 的甘氨酸。

39. 如权利要求 1、3 至 5、7、8 和 10 至 38 中任一项所述的组合物，其中所述组合物在真空下经历升华以产生冻干组合物。

40. 一种冻干组合物，其从如权利要求 1、3 至 5、7、8 和 10 至 38 中任一项所述的组合物冻干。

41. 一种复原的组合物，其包含在水溶液中复原的如权利要求 39 或 40 所述的冻干组合物。

42. 如权利要求 41 所述的复原的组合物，其中所述水溶液选自水、Tris EDTA(TE)、磷酸盐缓冲盐水 (PBS)、Tris 缓冲液或盐水。

43. 如权利要求 1、3 至 5、7、8 和 10 至 38 中任一项所述的组合物，其中所述组合物在以冻干形式在 50℃下存放至少 4 个月之后保持至少 80％、至少 90％或至少 95％的纯度。

44. 如权利要求 1、3 至 5、7、8 和 10 至 38 中任一项所述的组合物，其中所述组合物在以冻干形式在 40℃下存放至少 1 个月之后保持至少 80％、至少 90％或至少 95％的免疫原性。

45. 一种针对病原体对受试者接种的方法，其包括施用如权利要求 1 至 44 中任一项所述的组合物。

46. 一种针对病原体对受试者接种的方法，其包括向受试者施用从如权利要求 39 或 40 所述的冻干组合物复原的药物组合物。

47. 一种制造强效的基于明矾的冷冻疫苗的方法，其包括悬浮包含至少约 10％糖和吸附于铝佐剂的抗原的组合物以及以足以在发生沉降之前冷冻所述悬浮组合物的速率冷冻所述组合物。

48. 如权利要求 47 所述的方法，其中所述组合物含有至少约 15％的糖。

49. 如权利要求 47 所述的方法，其中所述组合物含有至少约 20％的糖。

50. 一种制备稳定的冻干组合物的方法，其包括将如权利要求 1、3 至 5、7、8 和 10 至 38 中任一项所述的组合物冻干，其中通过小噬细胞溶解分析 (MLA)、尺寸排阻色谱 (SEC-HPLC) 和 / 或阴离子交换色谱 (AEX-HPLC) 测量所述复原的冻干组合物的稳定性。

温度稳定性疫苗制剂

[0001] 政府权利

[0002] 本发明是部分地在政府扶持下依据许可 HHSO100201000059C 完成的。政府可以对本发明具有某些权利。

[0003] 以电子方式递交的序列表的参考

[0004] 与本申请一起提交的以电子方式递交的在 ASCII 文本文件（名称“2479115PC02_sequencelisting.txt”;大小 :12,954 字节 ;以及创建日期 :2013 年 6 月 25 日）中的序列表的内容以引用的方式整体并入本文中。

发明领域

[0005] 本发明涉及含有吸附于铝佐剂的抗原的温度稳定性疫苗制剂以及制备这些制剂的方法。本发明包括冻干和冷冻的疫苗制剂。本发明包括温度稳定性疫苗、制造温度稳定性疫苗的方法和使用方法。

[0006] 发明背景

[0007] 炭疽是一种熟知的由革兰氏阳性细菌炭疽杆菌 (Bacillus anthracis ; B.anthracis) 引起的传染病。在三种类型的炭疽感染（皮肤感染、胃肠感染和吸入性感染）中，皮肤炭疽最常见并且可用多种抗生素相对容易地治疗。另两种类型的炭疽感染是罕见的，但是即便在用积极的抗微生物疗法下通常也是致命的。

[0008] 主要的毒力因子炭疽毒素由以下三种蛋白质组成 :保护性抗原 (PA,83 千道尔顿，kDa)、水肿因子 (EF,89kDa) 和致死因子 (LF,90kDa)。毒素组分以 PA+EF（水肿毒素）和 PA+LF（致死毒素）的双重组合起作用。PA 是一种细胞受体结合蛋白并且将另两种蛋白质 (EF 和 LF) 递送到受感染细胞的细胞溶质中。

[0009] 最有效的预防炭疽的已知方法是接种。美国目前唯一的经 FDA 批准的炭疽疫苗（由 Emergent BioSolutions Inc. 以商标 BioThrax®（炭疽吸附疫苗）制造）是从来自无毒力炭疽杆菌 V770-NP1-R 菌株的无不育细胞的滤液制得。得到许可的炭疽疫苗也称为炭疽吸附疫苗（或 AVA)。所述疫苗主要由 PA 组成，并且将氢氧化铝用作佐剂。所述疫苗是在 20 世纪 50 和 60 年代期间开发出并且由 FDA 向 Emergent BioSolutions Inc 颁发许可。所述疫苗示出小于 0.06％的全身性反应。关于疫苗在人体内引发免疫反应的能力有较多的文献记录。目前许可 AVA 疫苗在 18 个月内用五剂，接着每年进行加强。

[0010] 虽然 AVA 疫苗有效且安全，但是正在开发用于制备使用重组技术来保护受试者免受致命性炭疽杆菌感染的疫苗的新型免疫原性组合物。因为保护性抗原 (PA) 是 LF 和 EF 起作用都需要的常见因子，所以其经常用于制备炭疽用疫苗。处于开发中的 PA 疫苗的实例包括美国专利 6,316,006 和 6,387,665 以及专利申请 US 2010/0183675、US2011/0229507 和 WO2010/053610 中所公开的那些。

[0011] 诸如 AVA 和 PA 的疫苗典型地含有至少一种佐剂以增强受试者的免疫反应。经常称为明矾的铝盐佐剂是目前最广泛使用的人用佐剂。明矾通常是氢氧化铝（也作为 ALHYDROGEL®（氢氧化铝）或磷酸铝销售）。用结合抗原的 ALHYDROGEL 配制 AVA

和“下一代”炭疽疫苗（如重组型 PA）。

[0012] 目前，含有明矾的疫苗需要冷链。全世界已经建立了冷链以将疫苗在存放和配送期间保持在 2-8℃下。维持冷链是昂贵且困难的。在冷链发生故障的情况下，疫苗可能暴露于高于或低于预期温度的温度。如果含有明矾的疫苗在货运和存放期间经历冷冻 / 解冻处理，那么通常建议将其丢弃。工业化国家和发展中国家都会发生冷链故障，并且冷链故障有许多原因，例如设备故障、资源缺乏或顺应性差。在许多发展中国家如印度尼西亚，在 75％的基线货运中记录到冷冻温度并且具有冷冻敏感性的冷冻是广泛的。参见 Hepatitis B vaccine freezing in the Indonesian cold chain ;evidence and solutions, Bulletin of the World Health Organization 2004 ;82 :99-105。

[0013] 将依赖于冷链的疫苗以及时的方式配送到有需要者也可能花费更长的时间。在生物恐怖事件或其它公共健康紧急事件的情况下，快速递送疫苗和其它医学对策的能力是重要的。消除对配送冷链的依赖使医学对策的递送在多种气候下更迅速和有效。

[0014] 为了避免或最小化冷链要求，将许多得到许可的疫苗配制成可在施用之前立即复原的干粉组合物。到目前为止，所有在美国许可使用的干粉疫苗都是通过冻干工艺制造的。冻干也称为冷冻干燥，是一种提高疫苗的长期稳定性的工艺。所述工艺涉及冷冻所述液体疫苗制剂并在真空下升华所述冷冻制剂。已经开发了其它技术如喷雾干燥和泡沫干燥以达到制造稳定的干粉疫苗的目的。这些较新的技术在不需要冷冻的情况下制得干粉疫苗物质并且可用于含明矾的疫苗。参见例如 Chen 等，2010，Vaccine 28 :5093-5099。然而，这些较新的技术仍在起步阶段并且有待在美国用于制造得到许可的疫苗。

[0015] 含明矾的疫苗组合物的冷冻（作为冻干工艺的部分或者用以制造冷冻疫苗）通常诱导铝粒子聚集并且引起吸附到明矾佐剂上的抗原降解，导致效力损失。另外，冷冻致使沉降的铝凝胶的高度降低（通常称为凝胶塌缩）。参见例如“The effect of freezing on the appearance, potency and toxicity of adsorbed and unadsorbed DPT vaccines,”1980, WHO Weekly Epidemiological Record 55 :385-92 ;“Temperature Sensitivity of Vaccines,”2006 年 8 月，WHO 出版物 WHO/IVB/06.10 ;Diminsky 等，1999，Vaccine，18(1-2) :3-17 ;Maa 等，2003，J Pharm Sci92(2) :319-332。

[0016] 因此，需要制造能耐冻的含明矾的疫苗。可以将这种疫苗冷冻，作为制造工艺（例如冻干或冷冻的疫苗）、货运过程或在存放期间的部分。本发明公开了用于制造含有明矾的温度稳定性疫苗的新型制剂。

[0017] 发明概述

[0018] 本发明提供含有明矾并且能够在效力极少降低至不降低的情况下冷冻的疫苗制剂。在一个实施方案中，冷冻疫苗组合物显示极少至无的冷冻所致的明矾凝胶塌缩。

[0019] 在一个实施方案中，疫苗或组合物包含至少 20％充当稳定剂的糖。在一个实施方案中，疫苗或组合物包含大于 15％、大于 20％、大于 25％或大于 30％的糖。在一些实施方案中，如果添加其它稳定剂如氨基酸和 / 或表面活性剂，那么可减少糖量而不削弱效力。对于冷冻和冻干的疫苗制剂，也可通过增大冷冻速率和通过冷冻悬浮粒子（如与沉降粒子相反）来提高效力。

[0020] 本发明包括冷冻疫苗组合物、冻干疫苗组合物（其经历冷冻，作为冻干工艺的部分）以及在货运和存放期间对冷冻 / 解冻条件不敏感的其它疫苗制剂。

[0021] 本发明的一些实施方案包括用于制备冻干疫苗的组合物，其包含至少一种吸附于铝佐剂的抗原和至少 20％ (w/v) 的非还原糖。另一实施方案包括一种温度稳定性液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原和至少 20％ (w/v) 的糖。另一实施方案包括组合物，其在冻干之前包含至少一种吸附于铝佐剂的抗原和至少 20％ (w/v) 的非还原糖，其中在复原之后，所述非还原糖是至少 6％ (w/v)。本发明的组合物可以进一步包含表面活性剂。在一些实施方案中，本发明的组合物包含至少一种吸附于铝佐剂的抗原、表面活性剂和至少 15％ (w/v) 的糖，其可用于制备冻干疫苗。

[0022] 本发明还包括温度稳定性液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂和至少 15％ (w/v) 的糖。在一些实施方案中，组合物进一步包含氨基酸。还包括稳定的液体疫苗组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂、氨基酸和至少 10％ (w/v) 的糖。

[0023] 本发明进一步提供用于制备冻干疫苗的组合物，其包含至少一种吸附于铝佐剂的抗原、表面活性剂、氨基酸和至少 10％ (w/v) 的糖。

[0024] 本发明可应用于众多含有吸附于铝佐剂的抗原的疫苗。在一个实施方案中，疫苗是炭疽疫苗如 rPA 或炭疽吸附疫苗。

[0025] 可以改变保护性抗原的来源。因此，在一些实施方案中，从不产孢子的炭疽杆菌细菌制造炭疽杆菌保护性抗原蛋白。在一些实施方案中，不产孢子的炭疽杆菌细菌是细菌的 ΔSterne-1(pPA102)CR4 菌株。在一些实施方案中，PA 蛋白在其它生物体如大肠杆菌 (E.coli) 中表达。

[0026] 在一些实施方案中，本发明的组合物可以进一步包含佐剂（例如，除铝以外）。

[0027] 本发明包括预防和治疗炭疽感染的方法，其包括向受试者施用药学上有效量的本发明的疫苗之一。在另一实施方案中，本发明包括在受试者体内诱导免疫反应的方法，其包括向受试者施用本发明的疫苗。

[0028] 本发明提供将疫苗冻干的方法，其包括 (i) 冷冻本发明的组合物和 (ii) 使冷冻的组合物升华。

[0029] 附图简述

[0030] 图 1. 与在 -80℃下冷冻并且解冻的相同组合物相比，在 2-8℃下的没有糖稳定剂的 rPA 疫苗的照片。

[0031] 图 2. 与在 -80℃下冷冻并且解冻的相同组合物相比，在 2-8℃下的具有 20％海藻糖和 2％精氨酸的 rPA 疫苗的照片。

[0032] 图 3. 在 2-8℃下和在 -80℃下接着解冻的有和无蔗糖的 rPA 疫苗的照片。

[0033] 图 4. 示出无糖 rPA 制剂在冷冻前后的 NF50 几何平均值的图以及示出明矾凝胶塌缩的照片。

[0034] 图 5. 示出未冷冻的有和无糖的冻干样本的 NF50 几何平均值反应的图。

[0035] 图 6. 示出含有 20％海藻糖的 rPA 组合物在冷冻 / 解冻前后 NF50 几何平均值的图。

[0036] 图 7. 示出与液体对照物相比，所有冻干样本的 NF50 几何平均值的图。

[0037] 图 8A-B. 示出冻干 rPA 在 (A)5℃和 50℃下与以线性 NF50 标度所示的 AVA 相比以及在 (B)5℃和 50℃下与以对数 NF50 标度所示的 AVA 相比随时间变化的 NF50 的图，每一疫

苗处于 1 ： 4 的稀释度下。

[0038] 图 9A-B. 示出冻干 rPA 在 (A)5℃和 50℃下与以线性 NF50 标度所示的 AVA 相比以及在 (B)5℃和 50℃下与以对数 NF50 标度所示的 AVA 相比随时间变化的 NF50 的图，每一疫苗处于 1 ： 16 的稀释度下。

[0039] 图 10A-B. 示出冻干 rPA 在 (A)5℃和 50℃下与以线性 NF50 标度所示的 AVA 相比以及在 (B)5℃和 50℃下与以对数 NF50 标度所示的 AVA 相比随时间变化的 NF50 的图，每一疫苗处于 1 ： 64 的稀释度下。

[0040] 图 11A-B. 示出在 5℃和 50℃下的冻干 rPA 和 AVA 在 (A) 第 35 天和 (B) 第 42 天时的 NF50 的比较的图，每一疫苗处于 1 ： 4 的稀释度下。

[0041] 图 12A-B. 示出在 5℃和 50℃下的冻干 rPA 和 AVA 在 (A) 第 35 天和 (B) 第 42 天时的 NF50 的比较的图，每一疫苗处于 1 ： 16 的稀释度下。

[0042] 图 13A-B. 示出在 5℃和 50℃下的冻干 rPA 和 AVA 在 (A) 第 35 天和 (B) 第 42 天时的 NF50 的比较的图，每一疫苗处于 1 ： 64 的稀释度下。

[0043] 图 14. 示出存放一个月的 rPA 液体疫苗 (液体 rPA F1) 相对于存放四个月的冻干疫苗 (LyoA、LyoB 和 LyoC) 随温度变化的 MLA(小噬细胞溶解分析)％值的比较的图。

[0044] 图 15A-B. (A) 相对于参考对照物，三种存放四个月的 rPA 冻干制剂 (LyoA、LyoB 和 LyoC) 与存放一个月的液体 rPA 相比随存放温度变化的 SEC 纯度％的相对下降的图。(B) 示出了 rPA BDS 参考标准的典型尺寸排阻色谱 (SEC-HPLC) 色谱图。

[0045] 图 16A-B. (A) 相对于参考对照物，三种存放四个月的 rPA 冻干制剂 (LyoA、LyoB 和 LyoC) 与存放一个月的液体 rPA 相比随存放温度变化的 AEX 纯度％的相对下降的图。(B) 示出了 rPA BDS 参考标准的阴离子交换色谱 (AEX-HPLC) 色谱图。

[0046] 图 17A-D. 示出在 5℃、25℃和 40℃下存放一个月的 LyoA 在剂量水平 0.25(A 图)、0.125(B 图)、0.0625(C 图) 和 0.03125(D 图) 下的 NF50 的比较的图。

[0047] 图 18A-D. 示出在 5℃、25℃和 40℃下存放一个月的 LyoB 在剂量水平 0.25(A 图)、0.125(B 图)、0.0625(C 图) 和 0.03125(D 图) 下的 NF50 的比较的图。

[0048] 图 19A-D. 示出在 5℃、25℃和 40℃下存放一个月的 LyoC 在剂量水平 0.25(A 图)、0.125(B 图)、0.0625(C 图) 和 0.03125(D 图) 下的 NF50 的比较的图。

[0049] 图 20A-D. 示出在 5℃、25℃和 40℃下存放一个月的液体 rPA 在剂量水平 0.25(A 图)、0.125(B 图)、0.0625(C 图) 和 0.03125(D 图) 下的 NF50 的比较的图。

[0050] 图 21. 示出 12 种如实施例 8 中所述的制剂的 NF50 值和平均数标准差的图。

[0051] 详述

[0052] 多年来，一直认为含明矾的疫苗不能冷冻。因此，不将含明矾的疫苗冷冻或冻干 (需要冷冻)，并且如果冷链中断引起冷冻，那么典型地将含明矾的液体疫苗丢弃。本发明的发明人得到激动人心的发现 ：当诸如海藻糖或蔗糖的糖构成炭疽疫苗组合物的约 20％ (w/v) 或更多时，组合物中的明矾不会因冷冻或解冻而塌缩。明矾塌缩是容易鉴别的并且与疫苗效力损失和粒子聚集相关。

[0053] 本发明人还鉴别了有助于防止和减少明矾凝胶塌缩的其它稳定化成分和工艺参数。例如通过向制剂中添加氨基酸，可减少防止明矾凝胶塌缩所需的糖量，例如减至约 10％ (w/v)。对明矾凝胶高度具有积极作用的工艺变化包括冷冻悬浮粒子 (而非沉降粒子) 和

增大冷冻速率。

[0054] 本文使用的章节标题仅用于组织目的并且不将其视为限制所述的主题。所有在本文中引用的文献或文献的部分（包括但不限于专利、专利申请、条款、书和论文）都以引用的方式整体明确并入本文中以达到任何目的。在一个或多个所并入的文献或文献的部分对术语的定义与本申请中对所述术语的定义相矛盾的情况下，以本申请中出现的定义为准。

[0055] 除非另外具体规定，否则使用单数包括复数。除非另外具体规定，否则措词“一种（个）(a 或 an)”意指“至少一种（个）”。除非另有说明，否则使用“或者”意指“和 / 或”。词语“至少一种（个）”的含义等效于词语“一种（个）或多种（个）”的含义。此外，使用术语“包括 (including)”以及其它形式，如“包括 (includes)”和“包括 (included)”是没有限制性的。此外，除非另外具体规定，否则术语如“要素”或者“组分”涵盖包含一个单位的要素或组分和包含大于一个单位的要素或组分二者。措词“约”意指在约 1 个单位内。

[0056] 本文所用的保护性抗原 (PA) 或重组型保护性抗原 (rPA) 是含有受体结合和移位结构域的炭疽毒素的组分（约 83kDa）。全长 PA 胺基酸序列的一个实例是：

[0057] EVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVVTSSTTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKSDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTEKGLDFKLYWTDSQNKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSQTRTISKNTSTSRTHTSEVHGNAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNANIRYVNTGTAPIYNVLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDDFSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIATYNFENGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPNGNLQYQGKDITEFDFNFDQQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG

[0058] (SEQ ID NO:1)。

[0059] SEQ ID NO:1 是 rPA102 的氨基酸序列，其从质粒 pPA102 表达。在 rPA102 从炭疽杆菌 ΔSterne-1(pPA102)CR4 分泌到细胞外间隙内的期间，前 29 个氨基酸（信号肽）被除去，得到 735 个氨基酸的成熟 rPA 蛋白 (82,674Da)。对成熟 rPA 序列加下划线 (SEQ ID NO：2)。

[0060] rPA102 氨基酸序列只是在本发明的范围内的一种具体炭疽蛋白的一个实例。来自多种炭疽菌株的 PA 蛋白（包括天然蛋白质）的其它氨基酸序列为本领域中已知的并且包括例如以下 GenBank 登记号：NP_652920.1、ZP_02937261.1、ZP_02900013.1、ZP_02880951.1，将其以引用的方式并入。还已知 PA 中用于降低其毒性或改善其表达特征的多种片段、突变和修饰，如在本说明书中别处所述的那些，多种融合蛋白也是如此。除非上下文或文本中明确表明排除那些形式，否则那些片段、突变体和融合蛋白包括于术语“PA”中。当有指示时，PA 片段、突变体和融合蛋白（无论具有全长 PA 或 PA 片段）是在毒素中和分析 (TNA) 中活性的引发抗血清的那些。

[0061] 本文所用的“温度稳定性”、“稳定”或“稳定性”是指在冷冻 / 解冻循环之后明矾凝胶的稳定性和疫苗的效力。本文所用的稳定疫苗是与保持在 2-8℃之间的相当的液体疫苗相比，在冷冻 / 解冻循环之后显示活性和 / 或效力和 / 或明矾凝胶塌缩和 / 或粒子聚集

不降低或者降低极少的疫苗。可使用本文所述的分析中的任一种或多种来测量稳定性，包括工作实施例，以及在本领域中已知的用于测量活性、效力和 / 或肽降解的分析。

[0062] 在某些实施方案中，可通过计算 50％中和因子 (NF50) 来测量抗原或疫苗例如保护性抗原的免疫原性。可基于来自给定数目的数据点的 NF50 值来计算疫苗制剂的 NF50 的几何平均数 (几何平均值或 <NF50>gm)。在某些实施方案中，通过使用来自标准毒素中和分析 (TNA)(Hering 等，Biologicals 32(2004)17-27 ;Omland 等，Clinical and Vaccine Immunology(2008)946-953 ; 和 Li 等，Journal of Immunological Methods(2008)333 : 89-106) 的血清样本，例如来自免疫小鼠或兔的血清测定 NF50 和 / 或几何平均值。使得毒素 50％中和的血清稀释度是“ED50”。由参考血清的 ED50 和测试血清的 ED50 的商计算每一测试血清相对于参考血清的中和能力 (50％中和因子或者 NF50，也称作中和比率)，即如下计算中和因子 NF50 :

[0063]

$$NF50 = \frac{ED50_{样本}}{ED50_{参考}}$$

[0064] 在某些实施方案中，可使用 T 检验或者单因素 ANOVA 以比较在 95％置信水平下来自不同制剂的 NF50 的几何平均数。在一个实施方案中，如果 NF50 几何平均值的 p 值大于 0.05，那么在制剂间，NF50 没有显著差异。在另一实施方案中，如果 p 值小于 0.05，那么在制剂间几何平均数 NF50 相互显著不同。

[0065] 由小鼠效力分析实验进行的中和因子 (NF50) 计算显示，NF50 值且因此效力与明矾凝胶塌缩相关。因此，可通过观察和测量已经在 -80℃下冷冻过夜，接着允许在室温下解冻的疫苗的明矾凝胶来评估稳定性。与对照疫苗 (仅存放在 2-8℃下的相同组合物) 相比，稳定的疫苗显示极少至无的明矾凝胶塌缩。可测量明矾凝胶高度并且可测定在冷冻 / 解冻样本和 2-8℃对照物之间的差异％。在一个实施方案中，约小于 1％、2％、3％、5％、8％、10％或 12％的差异表明疫苗稳定。

[0066] 也可通过在冷冻之后分析组合物的完整蛋白质 (例如与明矾一起完整的 rPA) 或相反地解吸附的蛋白质 (例如从明矾解吸附的 rPA) 来评估稳定性。例如，可通过 ELISA 通过分析和表征游离 rPA102(释放) 来测定稳定性 ;通过例如差示扫描量热法和固有荧光来测定蛋白质结构 ;通过 A_{280}来测定解吸附的游离蛋白质 ;通过 SDS-PAGE、SEC 和或 RP-HPLC 来测定纯度和骨架降解 ;通过 IEX 或等电位聚焦来测定电荷变化 ;并且通过小噬细胞溶解分析 (MLA) 来测定生化活性。

[0067] 在一个实施方案中，温度稳定性疫苗是在暴露于冷冻 / 解冻条件之后的疫苗 (例如冷冻疫苗或冻干疫苗)，显示与存放在约 2-8℃下的相当的液体疫苗相同，或者至少约 98％、至少约 95％、至少约 93％、至少约 90％、至少约 88％或至少约 85％相同的效力。在一个实施方案中，将炭疽小鼠效力分析用于测定冷冻或冻干的疫苗是否是强效的。

[0068] 在一些实施方案中，组合物在以冻干形式在 40℃下存放至少 1 个月之后保持至少 80％、至少 90％或至少 95％免疫原性。

[0069] 本发明的疫苗是温度稳定性疫苗。本发明的制剂稳定所在的温度通常低于约 30℃，但是可以高于 30℃、35℃、40℃、45℃或 50℃。在一些实施方案中，制剂的稳定性与低于约 25℃、约 20℃、约 15℃、约 10℃、约 8℃、约 5℃、约 4℃或约 2℃的温度有关。因此，在

一些实施方案中，温度在25℃至约-10℃、约20℃至约-10℃、约15℃至约-10℃、约10℃至约-10℃、约8℃至约-10℃、约5℃至约-10℃、约15℃至约-5℃、约10℃至约-5℃、约8℃至约-5℃和约5℃至约-5℃的范围内。

[0070] 实施例章节描述多种用于测定稳定性的方法。在一些实施方案中，与仅新鲜和/或5℃存放的相同样本相比，本发明的疫苗在冷冻解冻之后没有示出稳定性有统计上显著的降低。在一些实施方案中，与在5℃下存放1、2、3、4、5、6、912、18、24、30、36、42、48、54或60个月相比，本发明的疫苗在-80℃、-20℃、25℃、40℃和/或50℃下存放相同时间段之后没有示出稳定性、免疫原性、效力或其任何组合有统计上显著的降低。

[0071] 在一些实施方案中，通过小噬细胞溶解分析(MLA)、尺寸排阻色谱(SEC-HPLC)和/或阴离子交换色谱(AEX-HPLC)测量组合物的稳定性。

[0072] 在一些实施方案中，组合物在以冻干形式在50℃下存放至少4个月之后保持至少80％、至少90％或至少95％的纯度。

[0073] 本发明的疫苗组合物含有吸附于铝佐剂（明矾）的抗原和稳定化所述制剂必要量的糖。例如，当与已经维持在2-8℃之间和/或显示极少至无明矾凝胶塌缩的类似液体疫苗相比时，本文所公开的疫苗制剂在暴露于冷冻/解冻条件之后显示极少至无效力降低。

[0074] 铝佐剂（明矾）可为例如氢氧化铝、磷酸铝或硫酸铝。在一个实施方案中，佐剂是氢氧化铝（例如ALHYDROGEL）。铝的量可改变相当多，而对明矾凝胶的稳定性没有明显影响（换句话说，增大组合物中明矾的量不会提高明矾凝胶塌缩的可能性）。在本发明的一个实施方案中，疫苗组合物包含约1-10mg/ml氢氧化铝。在另一实施方案中，组合物包含约1.5至5mg/ml氢氧化铝。在另一实施方案中，疫苗组合物包含约1.5、2、2.5、3、3.5、4、4.5或5mg/ml氢氧化铝。

[0075] 据信本发明的稳定疫苗组合物可用以稳定化与明矾一起配制的任何抗原。例如，抗原可为炭疽杆菌重组型保护性抗原(rPA)或来自无毒力炭疽杆菌菌株如V770-NP1-R(例如炭疽吸附疫苗）的无细胞滤液。

[0076] 例如在颁予Park和Giri的美国专利号7,201,912、颁予Ivins等的美国专利号6,387,665、颁予Worsham等的美国专利号6,316,006和颁予Leppla等的美国专利号7,261,900（其各自以引用的方式整体并入）中描述了表达炭疽杆菌蛋白，包括PA（以及片段、突变体和融合蛋白）的方法。例如，如美国专利号7,201,912中所述，pBP103是全长野生型rPA的表达载体。来自pBP103的PA序列与野生型PA同一。

[0077] 本发明的一些实施方案包括包含在炭疽杆菌中表达（包括在形成孢子或不形成孢子的炭疽杆菌菌株或该两者中表达）的PA的制剂。例如，PA可来源于不形成孢子的炭疽杆菌菌株ΔSterne-1(pPA102)CR4（即rPA102）。参见例如都颁予Ivins等的美国专利号6,316,006和美国专利号6,387,665（其各自以引用的方式整体并入本文中）。一些本发明的组合物包含来自无毒力炭疽杆菌菌株V770-NP1-R的PA。

[0078] 本发明的制剂也可以包括由异源生物体表达的炭疽杆菌PA。例如，本发明包括在大肠杆菌中表达的PA。

[0079] 另外，还已经描述了多种PA片段、突变体和融合蛋白并且其可用于目前的制剂中。例如，可以对PA进行修饰以使其缺乏功能性结合位点，从而防止PA结合于天然PA所结合的炭疽毒素受体(ATR)（参见Bradley,K.A.,Nature(2001)414:225-229)或者结合于

天然 LF。举例来说，在结构域 4 的氨基酸残基 315-735 内部或附近或氨基酸残基 596-735 内部或附近进行修饰可以使 PA 不能结合于 ATR。或者（或另外），在大多数全长 PA 序列中见于残基 163-168 处或其周围的 PA 弗林蛋白酶裂解位点“RKKR”(SEQ ID NO:3) 可以通过在弗林蛋白酶裂解位点内部或附近进行缺失、插入或取代而失活。例如，天然 PA 的所有弗林蛋白酶裂解位点残基都可以缺失。其它突变 PA 包括二肽 Phe-Phe 已经修饰以使 PA 对糜蛋白酶具有抗性的那些。PA 片段或 PA 融合蛋白也可以是 PA 突变体。

[0080] PA 片段的特定实例包括美国专利号 7,201,912 中的那些，例如，由 pBP111 表达的 PA64、由 pBP113 表达的 PA47，由 pBP115 表达的 PA27。那些片段中的一些也可以包括突变以例如消除弗林蛋白酶裂解位点 RKK(SEQ ID NO:3) 或通过二肽序列 Phe-Phe(FF) 形成的糜蛋白酶敏感位点。另外，片段可以在 N 端包括一或两个其它氨基酸。涉及 PA 的融合蛋白的实例包括美国专利号 7,201,912 中的那些，例如由质粒 pBP107、pBP108 和 pBP109 表达的 PA-LF 融合蛋白。本发明还包括包含 HIS- 标签 PA 的制剂。然而当使用片段、突变体或融合蛋白时，通常需要所述片段、突变体或融合蛋白在小鼠、豚鼠或兔中的一个或多个中引发针对用例如 Ames 菌株的炭疽孢子的 LD_{50} 产生的攻击的保护性免疫。

[0081] 可使用来自重组型来源和 / 或非重组型来源的 PA 并且本发明的制剂会提高这些制备物的稳定性。

[0082] 在一个实施方案中，疫苗组合物包含约 75 至 750μg/ml、100 至 500μg/ml、100 至 250μg/ml、100 至 750μg/ml 或 250 至 750μg/ml 抗原，例如 rPA。例如，本发明包括包含约 150、200、250、300、350、400、450 和 500μg/ml 抗原例如 rPA 的疫苗。在一些实施方案中，疫苗包含约 175μg 抗原（例如 rPA)/1500μg 氢氧化铝。在一些实施方案中，疫苗包含约 200μg/ml 抗原（例如 rPA) 和约 0.5mg/ml 氢氧化铝。在其它实施方案中，疫苗包含约 250μg 抗原（例如 rPA)/100 至 250μg 氢氧化铝。在一些实施方案中，抗原是炭疽抗原，如保护性抗原。在一些实施方案中，保护性抗原与 SEQ ID NO:2 的多肽具有至少约 80％的同一性。一些本发明的组合物包含约 150-500μg/ml 保护性抗原或约 150、175、200、225、250、275、300、325、400、375、400、425、450、475 或 500μg/ml 保护性抗原。

[0083] 在一些实施方案中，本发明的组合物含有约 0.5 至 1.5mg/ml 氢氧化铝。在一些实施方案中，组合物含有约 0.5mg/ml 或约 1.5mg/ml 氢氧化铝。

[0084] 在一些实施方案中，铝佐剂选自氢氧化铝、磷酸铝和硫酸铝。

[0085] 本发明的炭疽疫苗无论是包含 rPA 或来自无毒力炭疽杆菌菌株的无细胞滤液的疫苗，都可向暴露于炭疽杆菌前或暴露于炭疽杆菌后的受试者施用。当暴露后施用时，疫苗可以结合抗生素施用。

[0086] 在另一实施方案中，抗原是基于蛋白质（例如重组型）的抗原，其选自乙型肝炎保护性抗原、肉毒梭菌神经毒素蛋白、单纯疱疹病毒抗原、流感抗原、先天性巨细胞病毒抗原、结核抗原、HIV 抗原、白喉抗原、破伤风抗原、百日咳抗原、葡萄球菌肠毒素 B(SEB) 和鼠疫耶尔森菌 (Yersinia pestis) 保护性抗原和 F1-V 融合蛋白。抗原可源于例如乳头状瘤病毒（例如 HPV)、流感、疱疹病毒、肝炎病毒（例如甲型肝炎病毒、乙型肝炎病毒、丙型肝炎病毒）、A、B 和 C 型脑膜炎球菌、B 型嗜血杆菌属流感 (HIB)、幽门螺旋杆菌 (Helicobacter pylori)、霍乱弧菌 (Vibrio cholerae)、链球菌属 (Streptococcus sp.)、葡萄球菌属 (Staphylococcus sp.)、肉毒梭菌 (Clostridium botulinum)、炭疽杆菌和鼠疫耶尔森菌。

[0087] 本发明的疫苗可在 -80℃下耐受冷冻过夜而无效力损失或明矾凝胶塌缩。本发明包括冷冻的液体疫苗以及冻干疫苗（本文中也称为冻结干燥的疫苗）。如本文所公开，冻干工艺包括液体组合物冷冻。接着对冷冻组合物进行冷冻下升华。对于冻干疫苗，所公开的疫苗组分和量意指接着受到冷冻的液体组合物而未必是干燥冻干饼或复原疫苗中的用量。由于干燥工艺，因此最终的冻干疫苗饼（干燥组合物）可以含有不同百分比的组分。

[0088] 本发明提供将疫苗冻干的方法，其包括 (i) 冷冻本发明的组合物和 (ii) 使冷冻的组合物升华。

[0089] 在一个实施方案中，本发明的疫苗包含约 20％或更多的玻璃成型剂如糖。在一个实施方案中，玻璃成型剂是还原糖，在一个实施方案中，疫苗包含非还原糖如海藻糖或蔗糖。在一个实施方案中，玻璃成型剂是海藻糖或蔗糖。如果将疫苗冻干，那么可能优选的是在冻干之前使用不超过约 40％的糖，因此疫苗形成饼状组合物。例如在冻干之前，疫苗可以包含约 15％、16％、17％、18％、19％、20％、21％、22％、23％、24％、25％、26％、27％、28％、29％、30％、35％或 40％糖。在一个实施方案中，例如在冻干之前，疫苗组合物包含约 10-40％、10-35％、10-30％、10-25％、10-20％、35-40％、30-40％、25-40％、20-40％、15-40％、20-30％、20-25％、25-30％、25-35％、21-40％、21-35％、21-30％ 21-25％或大于 10％、15％、20％、21％、22％、23％、24％、25％、26％、27％、28％、29％、30％、31％、32％、33％、34％、35％或 36％ (w/v) 糖。在一些实施方案中，例如在冻干之前，组合物含有大于约 15％、16％、17％、18％、19％、20％、21％、22％、23％、24％和 25％ (w/v) 糖。

[0090] 如本文所公开，本发明的发明人已经鉴别出包含至少约 20％海藻糖或蔗糖的明矾疫苗组合物可耐受冷冻 / 解冻条件。当添加某些其它稳定剂（例如表面活性剂和 / 或氨基酸）和 / 或将本文所公开的工艺改进并入（例如增大冷冻速率、冷冻悬浮粒子）时，可将糖量减至约 15％ (w/v) 或甚至约 10％ (w/v) 而不影响疫苗效力。

[0091] 在本发明的一个实施方案中，例如在冻干之前，包含吸附于铝佐剂的抗原和糖（例如 15％ w/v 或更多）的疫苗组合物也含有增溶剂如表面活性剂。在一个实施方案中，表面活性剂是非离子洗涤剂如聚山梨醇酯 80（TWEEN® 80）。在一个实施方案中，疫苗组合物包含在约 0.001％和约 0.05％之间的表面活性剂（如聚山梨醇酯 80）。在一个实施方案中，组合物包含约 0.020％、约 0.025％或约 0.020％至 0.025％ (w/v) 表面活性剂（如聚山梨醇酯 80）。可使用的其它表面活性剂包括但不限于聚山梨醇酯 20、普洛尼克 L68(pluronic L68)、聚氧乙烯 9-10 壬基酚 (Triton™ N-101，辛基酚 9)、Triton™ X-100 和脱氧胆酸钠。在一个实施方案中，在制造过程期间除去表面活性剂，因此最终药品中不存在表面活性剂。在一个实施方案中，在例如冻干工艺的冷冻期间存在表面活性剂。在一些实施方案中，本发明的制剂不包含表面活性剂。

[0092] 本发明人已经发现，如果例如在冷冻和 / 或冻干之前将氨基酸（例如丙氨酸、精氨酸、甘氨酸和脯氨酸）添加到组合物中，那么可以将糖的百分比减至多达约 10％ (w/v) 而对效力有极少至无影响和 / 或有极少至无明矾凝胶塌缩。添加到疫苗组合物中的氨基酸的量可改变。在一个实施方案中，疫苗组合物包含 0.5％至约 15％ (w/v) 的氨基酸或氨基酸组合。在一个实施方案中，疫苗组合物包含约 2-10％ (w/v) 的氨基酸或氨基酸组合。在本发明的一个实施方案中，疫苗包含约 2％精氨酸或丙氨酸。在本发明的另一实施方案中，疫苗包含约 10％甘氨酸。在一些实施方案中，疫苗组合物包含约 2-10％、2-8％、2-6％、2-4％、

2-3％、3-10％、5-10％、7-10％、2.5-5％、3-5％、3-7％或 4-6％ (w/v) 氨基酸或氨基酸组合。在一些实施方案中，存在两种、三种或更多种氨基酸，如选自丙氨酸、甘氨酸、脯氨酸和/或精氨酸。在一些实施方案中，组合物含有约 0.5-4％、1-4％、1.5-4％、2-4％、2.5-4％、3-4％、3.5-4％、0.5-1％、0.5-1.5％、0.5-2％、0.5-2.5％、0.5-3％、0.5-3.5％、0.5-4％、1-3％、1-2％、2-3％或 1.5-2.5％ (w/v) 丙氨酸或精氨酸。在一些实施方案中，组合物含有约 2％、1.75％、2.25％、2.5％、2.75％、3％、3.25％、3.5％、3.75％或 4％ (w/v) 丙氨酸或精氨酸。在一些实施方案中，组合物含有约 6-12％、7-12％、8-12％、9-12％、10-12％、11-12％、6-11％、6-10％、6-9％、6-8％、6-7％、7-11％、8-10％、7-10％、11-9％、7-8％、8-9％或 9-10％ (w/v) 甘氨酸。在一些实施方案中，组合物含有约 6％、7％、8％、9％、10％、11％或 12％ (w/v) 甘氨酸，在一些实施方案中，所述氨基酸浓度是在冷冻和/或冻干之前的。

[0093] 在一些实施方案中，制剂不包含氨基酸溶液或不含有除作为多肽抗原的部分的氨基酸以外的氨基酸。

[0094] 在一些实施方案中，所述制剂进一步包含一种或多种其它成分。例如，制剂可以包括一种或多种盐，如氯化钠、磷酸钠或其组合。一般来说，制剂中存在的每种盐为约 10mM 至约 200mM。

[0095] 疫苗制剂可以含有缓冲液如 20mM TRIS-HCL。也可以改变制剂的 pH 值。一般来说，其在约 pH 6.2 至约 pH 8.0 之间。在一个实施方案中，疫苗的 pH 值为约 7.4。

[0096] 在另一实施方案中，制剂进一步包含糖醇如山梨醇。在一个实施方案中，制剂包含 0.25％山梨醇。

[0097] 在一些实施方案中，本发明的组合物和疫苗制剂可以含有其它佐剂，例如免疫刺激序列 (ImmunoStimulatory Sequences；ISS，CpG) 和磷酸钙。对于 ISS，通常在最终蛋白质浓度 50μg/ml 下使用蛋白质样本。佐剂的其它非限制性实例包括但不限于：CGP7909(例如参见美国专利号 7,223,741，其以引用的方式整体并入本文中)、CpG1018(参见例如 2010/0183675，其以引用的方式整体并入本文中)、葡萄哌喃糖基脂质佐剂 (GLA)、PolyI PolyC(PIPC)、N-乙酰基-胞壁酰基-L-苏氨酰基-D-异谷氨酰胺 (thr-MDP)、N-乙酰基-降胞壁酰基-L-丙氨酰基-D-异谷氨酰胺 (CGP 11637，称为降 MDP)、N-乙酰基胞壁酰基-L-丙氨酰基-D-谷氨酰胺酰基-L-丙氨酸-2-(1′-2′-二棕榈酰基-sn-甘油基-3-羟基磷酰基氧基)-乙胺 (CGP 19840A，称为 MTP-PE)，和 RIBI，其含有三种从细菌提取的组分，即含单磷酰基脂质 A、海藻糖二梅菌酸酯和细胞壁骨架 (MPL+TDM+CWS) 的 2％角鲨烯/TWEEN 80 乳液。

[0098] 本发明包括包含以下制剂的组合物：0.15mg/mL 抗原、1.5mg/mL 铝、20％海藻糖、2％丙氨酸和 0.025％表面活性剂；0.5mg/mL 抗原、5mg/mL 铝、20％海藻糖、2％丙氨酸和 0.025％表面活性剂；0.5mg/mL 抗原、5mg/mL 铝、20％海藻糖、1％蔗糖、2％丙氨酸和 0.025％表面活性剂；和 0.5mg/mL 抗原、5mg/mL 铝、20％海藻糖、2％丙氨酸和 0.025％表面活性剂。在一些实施方案中，这些制剂中的抗原和/或表面活性剂分别是 PA 和 Tween® 80。在一些实施方案中，本发明的组合物包含 5mM NaPi(pH 7.0) 缓冲液或 20mM Tris(pH 7.4)。实施例中描述了本发明中包括的其它组合物。

[0099] 可制备本发明的疫苗以用作注射剂。组合物可为温度稳定性液体制剂(例如可耐

受冷冻/解冻循环）或冷冻组合物。所述组合物也可以用来制造可在施用之前例如用药学上可接受的载剂复原的冻干干粉疫苗。通常通过常规途径例如静脉内、皮下、腹膜内或经粘膜途径施用疫苗。可以通过非经肠注射，例如皮下或肌肉内注射来施用。

[0100] 在一些实施方案中，对本发明的组合物进行冷冻，接着在真空下升华以制造冻干组合物。

[0101] 术语“复原”是指使冻干形式恢复到液体形式，例如通过将之前为了保存和/或存放而改变的物质再水合，例如将已经存放的本申请的冻干 rPA 制剂恢复到液态而实现。可在产生适用于施用的稳定水溶液的任何水溶液中复原本申请的冻干组合物。这种水溶液包括但不限于无菌水、TE(Tris EDTA)、磷酸盐缓冲盐水 (PBS)、Tris 缓冲液或生理盐水。可用比用于冻干样本更低、相同或更高的体积复原冻干样本。

[0102] 应了解，可根据多种相关因素，包括待治疗病状、所选施用途径、个别患者的年龄、性别和体重以及患者症状的严重度来确定复原的本申请的冻干疫苗制剂的剂量，并且可用单次剂量、分次剂量或多次剂量施用。

[0103] 以与剂量制剂相容的方式并且以将预防和/或治疗有效的量施用疫苗。施用量取决于待治疗的受试者、受试者的免疫系统合成抗体的能力和所需的保护程度，其通常在每剂 5μg 至 500μg 抗原的范围内。在一个实施方案中，疫苗包含至少约 10μg PA、25μg PA、50μg PA、75μg PA、100μg PA、125μg PA、150μg PA、200μg PA、225μg PA、250μg、275μg、300μg PA。抗原的精确量取决于待递送的抗原。

[0104] 可以以单次剂量时程或任选地以多次剂量时程给予疫苗。可以例如以 0.5mL 剂量施用疫苗组合物。对于暴露前预防，在多次剂量时程中，初级接种过程可以有 1-6 个独立剂量，继之以在后续时间间隔下给予用于维持和或加强免疫反应的其它剂量，例如在 1-4 个月时的第二剂，以及需要时在几个月之后的后续剂量。

[0105] 对于暴露后预防，疫苗也可以根据单次剂量或多次剂量方案进行施用。例如，在一个实施方案中，在暴露后 0、2 和 4 周的时间分 3 剂施用疫苗。给药方案还将至少部分地根据个体需要、从业者判断和/或测试结果，例如对疫苗/抗原的免疫反应的测量水平如针对抗原的抗体水平和/或 T- 细胞活性来确定。

[0106] 另外，含有免疫原性抗原的疫苗可以结合其它免疫调节剂，例如免疫球蛋白、抗生素、介白素（例如 IL-2、IL-12）和/或细胞因子（例如 IFN-β、IFN-α）施用。

[0107] 在一个实施方案中，向对炭疽暴露后的受试者施用疫苗。在本实施方案中，可以与抗生素结合施用疫苗。可以与疫苗一起施用的抗生素包括但不限于青霉素 (penicillin)、多西环素 (doxycycline) 和环丙沙星 (ciprofloxacin)。

[0108] 本发明包括治疗（暴露后预防）或预防（暴露前预防）炭疽感染的方法，其包括向受试者施用药学上有效量的本发明的疫苗。在一个实施方案中，炭疽感染是吸入性炭疽（吸入炭疽）的后果。如本文所用，疫苗的药学上有效量是诱导免疫反应的量。在一个实施方案中，疫苗的药学上有效量是包含至少 25μg PA 的量。如本文所用，受试者是哺乳动物，如人。

[0109] 本发明还提供通过向受试者施用足以刺激免疫反应的量的本发明疫苗而在受试者体内刺激免疫反应的方法。在一个实施方案中，通过增大对疫苗中的抗原有特异性的抗体效价来测量免疫刺激。在其它实施方案中，通过对疫苗中的抗原具有特异性的细胞毒性

T 淋巴细胞中增大的频率来测量免疫刺激。

[0110] 还提供针对病原体对受试者接种的方法，其包括施用本发明的组合物。另外提供针对病原体对受试者接种的方法，其包括向受试者施用从本发明的冻干组合物复原的药物组合物。本发明进一步包括制造强效基于明矾的冷冻疫苗的方法，其包括悬浮包含至少约 10％、至少 15％、至少 20％、至少 21％、至少 25％或至少约 30％糖和吸附于铝佐剂的抗原的组合物以及以足以在发生沉降之前冷冻所述悬浮组合物的速率冷冻所述组合物，例如急骤冷冻。

[0111] 本发明的一些实施方案提供制备稳定冻干组合物的方法，其包括冻干本发明的组合物，其中通过小噬细胞溶解分析 (MLA)、尺寸排阻色谱 (SEC-HPLC) 和 / 或阴离子交换色谱 (AEX-HPLC) 测量复原的冻干组合物的稳定性。

[0112] 对于炭疽疫苗，可如多个实施例中所述测试制剂的免疫原性。例如，可用例如 10μg、20μg 或更多的悬浮于佐剂乳液中 rPA 使小鼠免疫。用在佐剂中乳化的盐水使对照小鼠免疫以用作阴性对照物。通常将小鼠免疫，接着以多个间隔，例如在免疫后第 0 天、第 21 天和第 28 天放血。接着例如通过 ELISA 分析血清的特异性抗体的存在，所述 ELISA 也可用于测定抗血清的效价。

[0113] 也可使用小鼠毒素中和抗体分析以测定炭疽疫苗制剂是否引发保护性抗体。在这个分析中，接着用 2 个致命剂量的致死毒素 (PA 和致死因子 (LF)) 腹膜内攻击用 rPA 免疫的小鼠。在攻击之后四天，对小鼠进行存活者评分。

[0114] rPA 制剂也可用于制备包含与炭疽毒素免疫反应的中和抗体的组合物。所得的抗血清可用于制造用于治疗炭疽暴露的药物。在本发明的一个实施方案中，抗体组合物包含纯化的抗 PA 抗体。“纯化的”意谓抗体基本上不含与其天然缔合的其它生物材料。本发明的纯化的抗体为至少 60 重量％纯、至少 70 重量％纯、至少 80 重量％纯、至少 90 重量％纯或至少 95 重量％纯。抗血清或从抗血清纯化的抗体也可用作检测 PA 片段或者天然蛋白质的诊断剂。

[0115] 通过增大冷冻速率将本发明的冷冻和冻干制剂制造成具有增大的效力。在一个实施方案中，将制剂急骤冷冻。

[0116] 也可通过冷冻悬浮而非沉降的组合物来增大效力。可通过轻微震荡来悬浮组合物并且将其立即冷冻。

[0117] 由以下实施例进一步阐明本发明，所述实施例旨在纯粹地例示本发明而决不限制本发明。

[0118] 实施例 1 :有和无海藻糖的液体 rPA 和 AVA 疫苗的冷冻 / 解冻

[0119] 如下表 1 中所概述制备有和无海藻糖的 rPA102 疫苗制剂。

[0120] 表 1. 用于冷冻 / 解冻分析的海藻糖制剂

[0121]

样本	rPA(mg/ml)	ALHYDROGEL (mg/ml)	缓冲液	pH	海藻糖	精氨酸	TWEEN 80
rPA 对照 1	0.15	1.5	20 mM TRIS-HCl	7.4	-	-	-
rPA 测试 1					20%	2%	0.025%

[0122] 在混配之后，将每一样本在10ml玻璃管中分成两个8ml等分试样。对于每一样本，将一管在轻微混合过夜之后放在-80℃下并且将另一管在轻微混合过夜之后放在2-8℃下。

[0123] 次日将在-80℃下存放过夜的样本在实验室工作台上解冻数小时（＞3-4小时），随后观察并且与变成室温的2-8℃样本比较。对样本拍照并且测量总的液体高度和ALHYDROGEL（氢氧化铝）高度。比较冷冻/解冻前后的常规的rPA102疫苗。图1是将在2-8℃下的rPA对照1样本（标记为5℃）与-80℃样本在解冻之后相比的照片。所述照片示出了经受冷冻/解冻条件的rPA对照1样本中的明矾凝胶显著塌缩。测试保护rPA102不受冷冻/解冻应激的常规制剂中的糖水平。如图1中所示，冷冻破坏的rPA102疫苗和效力(MRPT)数据与生理化学和凝胶高度（塌缩）相关。图2是将在2-8℃下的rPA测试1样本（标记为5℃）与-80℃样本在解冻之后相比的照片。解冻的rPA测试1样本中的明矾没有明显的塌缩。表2提供相对明矾高度%的概述。

[0124] 表2. 相对明矾高度%

[0125]

制剂	5℃	-80℃
rPA 对照 1	100%	32.6%
rPA 测试 1	100%	101.5%

[0126] 用含有15%海藻糖、0.15mg rPA/mL、2%丙氨酸、0.025%聚山梨醇酯80、25mM NaPi(pH 7.4)的测试组合物进行类似的冷冻/解冻实验，与无15%海藻糖的对照制剂相比，得到类似的结果（数据未示出）。

[0127] 将一小瓶BioThrax®（炭疽吸附疫苗）、AVA和一小瓶AVA+25%海藻糖在轻微混合之后放到-80℃下。将第二小瓶BioThrax和第二小瓶AVA+25%海藻糖在轻微混合之后放在2-8℃下过夜。次日，使-80℃小瓶解冻并且检查所有小瓶。铝凝胶高度看似与存放在2-8℃下的BioThrax和两个含有25%海藻糖的AVA样本大约相同。铝凝胶高度比存放在-80℃下的BioTlirax（无海藻糖）低得多。（数据未示出）。

[0128] 实施例2：有和无蔗糖的液体rPA疫苗的冷冻/解冻

[0129] 如下表3中所概述制备有和无蔗糖的rPA102疫苗制剂。

[0130] 表3. 用于冷冻/解冻分析的蔗糖制剂

[0131]

样本	rPA(mg/ml)	ALHYDROGEL (mg/ml)	缓冲液	pH	蔗糖
rPA 对照 2	0.5	5	20 mM TRIS-HCl	7.4	-
rPA 测试 2					10%

[0132] 在混配之后，将每一样本在 10ml 玻璃管中分成两个 10ml 等分试样。对于每一样本，将一管在轻微混合过夜之后放在 -80℃下并且将另一管在轻微混合过夜之后放在 2-8℃下。

[0133] 次日将在 -80℃下存放过夜的样本在实验室工作台上解冻 2-3 小时，随后进行观察。图 3 含有来自 2-8℃（标记为 5℃）和 -80℃的每一制剂在都达到室温之后的照片。如所示，与仍冷藏在 2-8℃下的样本相比，在解冻之后，在两个 -80℃样本 (rPA 对照 2 和 rPA 测试 2) 中都发生凝胶塌缩。然而，在不包括蔗糖的 rPA 对照 2 样本中凝胶塌缩的量明显更大。

[0134] 实施例 3：体内小鼠效力分析

[0135] 如表 4 中所概述制备冻干疫苗。用注射用水将经过干燥的疫苗复原至最终 rPA 浓度 0.15mg/ml(75μg/0.5ml 剂量)，接着在生理盐水中稀释 10 倍以得到 0.1(DL) 的剂量水平。

[0136] 将 5-8 周龄并且重 20-25 克的雌性 CD-1 小鼠各自用于本研究中。将 0.1DL 疫苗 (0.5ml) 腹膜内注入 20 只雌性 CD-1 小鼠的组中，并且在第 28 天收集血清以便在小鼠中在毒素中和分析 (TNA) 中评估其中和炭疽 LT 细胞毒性的能力。

[0137] 表 4. 用于小鼠效力分析的冻干制剂

[0138]

样本	制剂
1 号冻干物	10%海藻糖、0.5 mg/ml rPA、5.0 mg/ml 铝、0.25%山梨醇、75 mM NaCl、1%精氨酸、20 mM Tris-HCL, pH 7
2 号冻干物	无糖、0.15 mg/ml rPA、1.5 mg/ml 铝、20 mM Tris-HCl, pH 7.4
3 号冻干物	30%海藻糖、0.15 mg/ml rPA、1.5 mg/ml 铝、2%精氨酸、0.025%聚山梨醇酯 80、20 mM Tris-HCl, pH 7.4
4 号冻干物	20%海藻糖、0.15 mg/ml rPA、1.5 mg/ml 铝、10%甘氨酸、0.025%聚山梨醇酯 80、20 mM Tris-HCl, pH 7.4

[0139] 通过计算中和因子 (NF50) 研究 rPA102 制剂的免疫原性。中和因子 NF50 定义如下：

[0140]

$$NF50 = \frac{ED50_{样本}}{ED50_{参考}}$$

[0141] 其中通过使用存放在 -20℃或低于 -20℃下的合格血清参考标准来制备有效剂量 50％ (ED50) 参考标准。

[0142] 基于来自 20 只小鼠的 20 个 NF50 值计算每一疫苗制剂的 NF50 的几何平均数（几何平均值）。将 T 检验或单因素 ANOVA 用于在 95％置信水平比较每一制剂的 NF50 的几何平均数。如果几何平均数 NF50 的 p 值大于 0.05，那么在制剂间 NF50（效力）没有显著差异。如果 p 值小于 0.05，那么制剂的几何平均数 NF50 相互显著不同。

[0143] 对照物 (2 号冻干物）是无稳定剂的 rPA102 制剂 (0.15mg/ml rPA、1.5mg/ml 铝、20mM Tris-HCL，pH 7.4)。图 4 中示出对于常规 rPA102 制剂，冷冻对来自相对小鼠效力分析的 NF50 几何平均值的影响。对照制剂对冷冻 / 解冻破坏敏感，在冻结过程之后免疫原性显著下降（图 4)。免疫原性的下降对应于溶液中铝凝胶高度的降低。

[0144] 相对于 3 号和 4 号冻干样本（其分别含有 30％和 20％海藻糖），1 号和 2 号冻干样本示出显著更低的免疫原性效力。图 7 中的 NF50 几何平均值结果示出了制剂中糖对 rPA102 疫苗冻干的影响。1 号冻干样本 (10％糖）不能保护 rPA102 免受冷冻干燥应激（参见图 7)。这些结果示出 20％和 30％糖能够保护 rPA102 免受冻干应激。凝胶塌缩的出现与效力损失相关。

[0145] 从另外两个不同之处仅在于不存在或存在海藻糖的制剂确定 NF50 反应 :1) 号：0.15mg/mL rPA、1.5mg/mL 明矾、2％精氨酸、0.025％ TWEEN 80（聚山梨醇酯 80)、20mM Tris-HCL(pH 7.4) 以及 2 号和 3) 号 :0.15mg/mL rPA、1.5mg/mL 明矾、20％海藻糖、2％海藻糖、0.025％ TWEEN 80（聚山梨醇酯 80)、20mM Tris-HCL(pH 7.4)，并且图 5 中示出在冷冻之前糖对 rPA102 的几何平均值的影响。在图 5 中，2 号和 3 号制剂组仅是相同制剂的不同小瓶。添加海藻糖对制剂的免疫原性没有影响，如由有和无糖并且不冷冻的两种制剂 (3 个样本）的 NF50 没有统计变化所证明（图 5)。图 6 中示出在这些含有海藻糖的制剂冷冻前后

几何平均值的比较。当使用这种制剂时，在冷冻前后免疫原性 (NF50) 没有统计上显著的变化（图 6）。另外，这种制剂在冷冻之后明矾凝胶没有塌缩（凝胶高度得到维持）（图 6 中的照片）。这些结果显示，含 20％海藻糖的测试制剂会保护这种 rPA 疫苗免受冷冻 / 解冻应激。

[0146] 实施例 4 ：兔免疫原性和稳定性研究

[0147] 在新西兰白 (NZW) 兔中使用毒素中和抗体分析 (TNA) 将在 5℃和 50℃下存放 4 个月的重组型保护性抗原 (rPA) 冻干疫苗制剂的免疫原性与炭疽吸附疫苗 (AVA)（BioThrax®）相比。这种兔免疫原性研究利用两种免疫时程（第 0 天和第 28 天），并且在第 -1 或 0、14、21、28、35、42、56 和 70 天采血。

[0148] 使用表 5 中所示的成分来制备 rPA 冻干疫苗。在冻干之前且在复原之后将最终制剂的成分共混，如表 5 中所示。简言之，将 2mL 悬浮液装在 10mL 玻璃小瓶中。使用 VirTis AdVantage 冻干器进行冻干。在冻干之后，将疫苗存放在 5℃和 50℃下。

[0149] 表 5. rPA 制剂

[0150]

成分	冻干前(2 mL悬浮液在10个小瓶中)	通过添加6.11 mL水至最终体积6.67 mL来复原之后
rPA, mg/mL	0.5	0.15
铝, mg/mL	5	1.50
海藻糖%	25%	7.5%
山梨醇	0.25%	0.075%
TWEEN 80	0.03%	0.0075%
精氨酸	1%	0.30%
NaCl, mM	75	22.5
Tris-HCl, mM pH 7.4	20	6.0
体积, mL	2	6.67

[0151] 具体来说，制备储备溶液并且（除 TWEEN 80 和 NaCl 以外）使用 0.1N NaOH 和 / 或 0.1N HCl 将 pH 值调节到 7.4。在制备储备溶液之后，在 200mL Nalgene 瓶中制备 150mL 以下制剂共混物 ：0.5mg/mL rPA、5.0mg/mL 铝、30％海藻糖 (w/v)、0.25％山梨醇 (w/v)、1％精氨酸 (w/v)、0.025％ TWEEN 80、75mm NaCl、20mM Tris-HCl(pH 7.4)，将其用于与 2mL 制剂共混物一起装在 10mL 小瓶中。

[0152] 在装在小瓶中之后，使用 VirTis AdVantage 冻干器，用以下程序干燥样本 ：

[0153] 初始冷冻 ：

[0154]

步骤	方法	设定
冷冻	冷冻(℃)	-60
	额外冷冻时间(分钟)	0
	冷凝器(℃)	-80
	真空(毫托)	90

[0155] 干燥：

[0156]

步骤	温度 ℃	时间 (分钟)	R/H	真空 毫托
1	-60	120	H	90
2	-28	60	R	
3	-28	1250	H	
4	-28	550	H	
5	25	480	R	
6	25	600	H	
7	30	120	R	
8	30	300	H	
9	35	120	R	
10	35	300	H	
11	40	120	R	
12	40	300	H	
13	45	120	R	
14	45	255	H	

[0157] 后干燥：

[0158]

二级干燥设定点温度	+65℃
后加热设定	
温度(℃)	+25
时间(分钟)	1250
真空(毫托)	1250

[0159] 如表 6 中所述存放小瓶。在免疫当天，将 6.11mL 无菌注射用水添加到每一小瓶中以使冻干样本复原。上下颠倒地混合小瓶直到所有制剂组分都完全溶解为止。在无菌生理

盐水中制备每一测试和对照物品的稀释物（1 ： 4、1 ： 16 和 1 ： 64）。

[0160] 将 NZW 兔用于本研究。NZW 兔常用作炭疽杆菌疾病的动物模型以测试毒性、免疫原性和功效，并且将 NZW 兔视为充分表征的模型，因为其具有与人中所见类似的发病机制和临床呈现（EK Leffel 等，Clin Vaccine Immunol. 19(18)：1158-1164，2012；AJ Phipps 等，Microbiol Mol Biol Rev. 68(4)：617-29，2004）。在第 0 天和第 28 天，使每一组 NZW 兔（每个疫苗组 10 只）接受冻干 rPA 疫苗制剂或 AVA 的具有 1 ： 4、1 ： 16 和 1 ： 64 稀释度的 0.5mL 肌肉内注射。AVA（BioThrax®）是包括 83kDa 保护性抗原蛋白的液体炭疽疫苗，并且用 1.2mg/mL 铝（以含氢氧化铝的 0.85％氯化钠的形式添加）、25mg/mL 苄索氯铵和 100mg/mL 甲醛（添加作为防腐剂）来配制。

[0161] 在第 -1 或 0、14、21、28、35、42、56 天并且在第 70 天结束之前收集血清样本。通过使用在第 -1 或 0、14、35 和 42 天收集的血清来进行 TNA 分析。表 6 概括了研究设计。

[0162] 表 6. 兔免疫原性研究设计

[0163]

组号	测试疫苗	疫苗稀释度	免疫时程（研究天数）	剂量体积（mL）	采血*	动物（兔）数目
1	AVA（阳性对照物）	1:4	第 0 天和第 28 天	0.5 mL	在剂量起始日之前（第 -1 天或第 0 天）以及在第 14、21、28、35、42、56 和 70 天	10（5M/5F）
2		1:16				
3		1:64				
10	在 5℃下冻干 4 个月的 rPA	1:4				
11		1:16				
12		1:64				
13	在 5℃下冻干 4 个月的 rPA	1:4				
14		1:16				
15		1:64				
* 来自测试第 -1 或 0、14、35 和 42 天的血清						

[0164] TNA 分析是一种功能测试，其评价使 LF 和 PA 的致命性炭疽杆菌毒素复合物（致死毒素，LT）失活所需的抗体的量。通过使用细胞毒性作为分析终点来将测试血清样本体外中和致死毒素的能力与标准血清样本的所述能力相比（PR Pittman 等，Vaccine 24(17)：3654-60，2006）。

[0165] 简言之，在烧瓶中在含有 4.5g/ 升 d- 葡萄糖并且补充有 10％热灭活牛血清、2mM L- 谷氨酰胺、1mM 丙酮酸钠、青霉素（50U/ml）、链霉素（50 μ g/ml）和 0.11mM 碳酸氢钠的杜贝可氏改良的伊格尔培养基（Dulbecco′ s modified Eagle media；DMEM）中培养 J774A.1 细胞 48 至 72 小时。收集细胞并且将其以 30,000 个细胞 / 孔接种在 96 孔组织培养板中，

接着培育 16 至 24 小时。在独立的 96 孔微量滴定板中以 2 倍稀释度制备血清样本，每个样本总共七个稀释度。接着将血清样本与恒定浓度的 LT(100ng/ml PA 和 80ng/ml LF) 一起培育 1 小时。接着将血清样本与 LT 一起添加到含有细胞的组织培养板的相应孔中并且培育四小时，此后添加 25μl/ 孔的 5mg/ml 四唑鎓盐 3-(4,5- 二甲基噻唑基 -2)-2,5- 二苯基四唑鎓溴化物 (MTT)。在培育 1 小时之后，通过使用 100μl/ 孔的 pH 值经使用 HCl 调节到 4.7 的酸化异丙醇 (50％ N,N- 二甲基甲酰胺 (含去离子水) 和 20％ SDS(200g 在 1 升 50％二甲基甲酰胺中)) 溶解细胞。将分析板再培育 16-24 小时，在 570 和 690nm 下测量吸光度，其中使用经校正的 Molecular Devices VersaMax Plate Reader 从 570 值减去 690 光学密度值。使用 SoffMax Pro 软件 (5.4.1 版，Sunnyvale,CA) 确定 ED50，ED50 为在光学密度测量中在 50％群体中产生数量效应的剂量。

[0166] 使用小鼠参考血清 (批号 MS011211) 的 ED50 来确定测试血清样本和阳性对照物的 NF50 值 (NF50 ＝ ED50 测试 /ED50 参考)。通过用含有 CPG 7909 佐剂的 AVA 疫苗使 300 只小鼠免疫来制备小鼠参考标准。从所述 300 只小鼠收集血清，汇集并冷冻存放在 -80℃下作为参考标准。表 7A-C 示出了分别来自第 14、35 和 42 天的血清样本的存放在 5℃下的冻干 rPA、存放在 50℃下的冻干 rPA 和存放在 5℃下的 AVA 对照物的平均动力学数据 (NF50) 的比较。

[0167] 表 7A. 第 14 天平均动力学数据 (NF50) 的比较

[0168]

样本存放 稀释度	rPA lyo 5°C 1:4	rPA lyo 50°C 1:4	AVA 5°C 1:4	rPA lyo 5°C 1:16	rPA lyo 50°C 1:16	AVA 5°C 1:16	rPA lyo 5°C 1:64	rPA lyo 50°C 1:64	AVA 5°C 1:64
平均值	0.10	0.13	0.05	NRS	NRS	NRS	0.02	0.02	NRS
标准差	0.07	0.08	0.02	NRS	NRS	NRS	0.01	0.01	NRS
CV%	0.72	61%	50%	NRS	NRS	NRS	31%	26%	NRS
几何平均值	0.07	0.11	0.04	NRS	NRS	NRS	0.02	0.02	NRS

[0169] 表 7B. 第 35 天平均动力学数据 (NF50) 的比较

[0170]

样本存放 稀释度	rPA lyo 5°C 1:4	rPA lyo 50°C 1:4	AVA 5°C 1:4	rPA lyo 5°C 1:16	rPA lyo 50°C 1:16	AVA 5°C 1:16	rPA lyo 5°C 1:64	rPA lyo 50°C 1:64	AVA 5°C 1:64
平均值	3.69	3.25	1.80	1.86	1.28	0.79	0.26	0.14	0.06
标准差	2.19	1.47	0.85	0.82	0.99	0.46	0.25	0.11	NRS
CV%	59%	45%	47%	44%	78%	59%	96%	77%	0%
几何平均值	3.14	2.98	1.61	1.70	0.94	0.67	0.16	0.10	0.06

[0171] 表 7C. 第 42 天平均动力学数据 (NF50) 的比较

[0172]

样本存放 稀释度	rPA lyo 5°C 1:4	rPA lyo 50°C 1:4	AVA 5°C 1:4	rPA lyo 5°C 1:16	rPA lyo 50°C 1:16	AVA 5°C 1:16	rPA lyo 5°C 1:64	rPA lyo 50°C 1:64	AVA 5°C 1:64
平均值	2.54	2.35	1.15	1.21	0.78	0.46	0.17	0.12	0.05
标准差	1.74	1.18	0.60	0.67	0.60	0.26	0.17	0.08	0.04
CV%	68%	50%	52%	56%	77%	56%	97%	64%	75%
几何平均值	2.07	2.13	1.01	1.08	0.57	0.40	0.11	0.10	0.04

[0173] NRS ＝无反应者

[0174] 在第 0 天，所有动物的毒素中和活性都测试为阴性。在所有三种剂量水平的所有

三种测试疫苗的<NF50>都在第35天时达到最大含量（图8A-B、9A-B和10A-B），显示rPA在所有三种剂量下皆具有与AVA类似或更好的免疫原性动力学。如图8A-B中所示，存放在5℃和50℃下的冻干rPA(1 ： 4稀释度）都具有高于AVA(1 ： 4稀释度）的<NF50>。类似地，在1 ： 16和1 ： 64的稀释度下，冻干rPA的<NF50>高于相当的AVA稀释度（参见图9A-B和10A-B)。

[0175] 对于在第35天时的1 ： 4稀释度，发现存放在5℃和50℃下的冻干rPA的<NF50>几何平均值(gm)分别为3.14和2.98；并且在1 ： 4稀释度下的AVA的<NF50>gm为1.61。在存放在5℃相对于50℃下的冻干rPA制剂(1 ： 4)之间，<NF50>gm没有统计差异，p ＝ 0.82(t检验)。发现组合数据(rPA lyo 5和50℃）的<NF50>gm是3.06；并且组合<NF50>gm在统计上高于AVA参考的组合<NF50>gm(1.61)，p ＝ 0.034(t检验)。在第42天(1 ： 4)，在存放在5℃相对于50℃下的rPAlyo之间，<NF50>gm没有统计差异（分别为2.07和2.13)，p ＝ 0.92(t检验）；并且发现组合<NF50>gm是2.10。2.10的组合<NF50>gm在统计上高于AVA参考的<NF50>gm(1.01)，p ＝ 0.028(t检验)。

[0176] 对于在第35天时的1 ： 16稀释度，发现存放在5℃和50℃下的冻干rPA的<NF50>gm分别为1.70和0.94。在存放在5℃和50℃下的rPA lyo之间，<NF50>gm没有统计差异，p ＝ 0.081(t检验)。发现组合<NF50>gm为1.27，并且rPA lyo(5℃和50℃）和AVA参考(0.67)的组合<NF50>gm没有统计差异，p ＝ 0.064(t检验)。在第42天(1 ： 16)，在存放在5℃相对于50℃下的rPA lyo之间，<NF50>gm没有统计差异（分别为1.08和0.57)，p ＝ 0.071(t检验）；并且发现组合<NF50>gm是0.78。0.78的组合<NF50>gm在统计上高于AVA参考的<NF50>gm(0.40)，p ＝ 0.05(t检验)。

[0177] 对于在第35天时的1 ： 64稀释度，发现存放在5℃和50℃下的冻干rPA的<NF50>gm在统计上不同（分别为1.06和0.10)，p ＝ 0.386(t检验)。发现组合<NF50>gm为0.13并且AVA参考为0.06。在第42天(1 ： 64)，在存放在5℃和50℃下的rPAlyo之间，<NF50>gm没有统计差异（分别为0.11和0.10)，p ＝ 0.91(t检验）；并且发现组合<NF50>gm是0.11。在0.11的组合<NF50>gm和AVA参考的组合<NF50>gm(0.04)之间没有统计差异，p ＝ 0.35(t检验)。

[0178] 与在第35和42天时在三种稀释度(1 ： 4、1 ： 16和1 ： 64)下的AVA相比，存放在5℃和50℃下的冻干rPA的NF50的几何平均数(<NF50>gm)示于图11A-B、12A-B和13A-B中。

[0179] 在第35天，发现存放在50℃下的冻干rPA疫苗的<NF50>gm在剂量1 ： 4、1 ： 16和1 ： 64下分别是2.98、0.94和0.1。在第35天，存放在5℃下的冻干rPA疫苗的<NF50>gm类似（分别为3.14、1.7和0.16)，与50℃(alph ＝ 0.05)相比没有统计上显著的差异。在第42天发现类似的结果。这些数据显示在50℃下存放4个月的冻干rPA疫苗的免疫原性并不显著不同于存放在5℃下的冻干rPA疫苗。

[0180] 在第35天，发现在剂量1 ： 4、1 ： 16和1 ： 64下，组合<NF50>gm(5℃和50℃）分别是3.06、1.27和0.1；并且在1 ： 4和1 ： 16下，与AVA相比，组合<NF50>gm的p值分别是p ＝ 0.034和p ＝ 0.064。发现组合<NF50>gm不低于（统计上高于或无差异)AVA的组合<NF50>gm。在第42天显示类似的结果。在第42天，1 ： 4、1 ： 16和1 ： 64的组合<NF50>值分别是2.10、0.78、0.11；并且在1 ： 4、1 ： 16和1 ： 64下，与AVA相比，组

合 <NF50>gm 的 p 值分别是 p = 0.92、p = 0.071 和 p = 0.91。这些免疫原性数据示出，在 5℃和 50℃下存放 4 个月的 rPA 冻干疫苗至少与 AVA 疫苗具有一样的免疫原性。

[0181] 总之，这些结果示出所测试的冻干制剂能够使 rPA 疫苗在 50℃下稳定至少 4 个月。数据显示，rPA 冻干制剂与 AVA 疫苗相比具有优越的热稳定性概况。因此，结果示出 rPA 冻干制剂对 rPA 炭疽疫苗存放有效，并且所测试的制剂室温稳定并且能够规避冷链配送。

[0182] 实施例 5：豚鼠免疫原性研究

[0183] 表 8 中概述了豚鼠免疫原性研究。

[0184] 表 8. 豚鼠免疫原性研究设计

[0185]

疫苗	稀释前	稀释	所需 GP(n) 315 g 至 385 g	报告的结果
AVA 参考批次	无	1/1.6	6 只雄性+6 只雌性(12)	存活数/总数
		1/4	6 只雄性+6 只雌性(12)	存活数/总数
		1/10	6 只雄性+6 只雌性(12)	存活数/总数
		1/25	6 只雄性+6 只雌性(12)	存活数/总数
新鲜 rPA102	无	1/1.6	6 只雄性+6 只雌性(12)	存活数/总数
		1/4	6 只雄性+6 只雌性(12)	存活数/总数
		1/10	6 只雄性+6 只雌性(12)	存活数/总数
		1/25	6 只雄性+6 只雌性(12)	存活数/总数
在 5℃下 12-15 个月的 rPA102 液体	无	1/1.6	6 只雄性+6 只雌性(12)	存活数/总数
		1/4	6 只雄性+6 只雌性(12)	存活数/总数

[0186]

		1/10	6 只雄性+6 只雌性(12)	存活数/总数
		1/25	6 只雄性+6 只雌性(12)	存活数/总数
在 40℃下冻干 1 个月(接着复原)的 rPA102	无	1/1.6	6 只雄性+6 只雌性(12)	存活数/总数
		1/4	6 只雄性+6 只雌性(12)	存活数/总数
		1/10	6 只雄性+6 只雌性(12)	存活数/总数
		1/25	6 只雄性+6 只雌性(12)	存活数/总数
攻击制备物				
每 0.1 mL 剂量的菌落形成单位	40		4 只雄性+4 只雌性(8)	死亡数/总数
接种动物总数			200 (100 只雄性和 100 只雌性)	

[0187] 实施例 6：小鼠中的免疫原性和生理化学稳定性

[0188] 与液体 rPA 疫苗相比测试三种冻干 rPA 疫苗制剂的免疫原性和生理化学稳定性。通过巨噬细胞溶解分析 (MLA)、尺寸排阻色谱 (SEC-HPLC) 和阴离子交换色谱 (AEX-HPLC) 测量 rPA 疫苗的含量和纯度（生理化学稳定性性质）。通过用四个剂量水平对 CD-1 小鼠接种并测试小鼠血清中和炭疽毒素的能力 (NF50) 来评价 rPA 疫苗的免疫原性。

[0189] 液体 rPA 疫苗制剂

[0190] 在无菌条件下在 0.15mg/mL rPA、1.5mg/mL 明矾、2％丙氨酸、0.01％ TWEEN 80、

25mM NaPi(pH 7.0)下制备液体 rPA 制剂(F1)。用于稳定性分析的样本含有装在 10mL 玻璃小瓶中的 5mL 液体悬浮液(每个小瓶 10 剂)。在肌肉内注射情况下预期的人用剂量是每 0.5mL 有 75μg rPA/750μg 明矾。

[0191] 冻干 rPA 疫苗制剂

[0192] 制备三种冻干的 rPA 制剂。表 9 中示出在冻干之前的最终制剂。用 0.15mg/mL rPA、1.5mg/mL 明矾、20%海藻糖、2% Ala、0.025% Tw80 和 5mM NaPi(pH 7.0)配制第一批次(lyoA)。第二和第三批次(分别是 lyoB 和 lyoC)含有高 3.3 倍的 rPA 和明矾浓度(分别是 0.5mg/mL 和 5.0mg/mL),糖、氨基酸和缓冲剂的变化微小,如表 9 中所示。

[0193] 表 9. 在冻干之前共混的最终制剂的浓度

[0194]

批号	rPA (mg/mL)	明矾 (mg/mL)	糖	氨基酸	TWEEN 80	缓冲液	液体 Lyo 体积, mL
LyoA	0.15	1.5	20%海藻糖	2%丙氨酸	0.025%	5 mM NaPi, pH 7.0	2 mL 装在 10 mL 小瓶中
LyoB	0.5	5	20%海藻糖	2%丙氨酸	0.025%	5 mM NaPi, pH 7.0	2 mL 装在 10 mL 小瓶中
LyoC	0.5	5	20%海藻糖+ 1%蔗糖	2%甘氨酸	0.025%	20 mM Tris, pH 7.4	2 mL 装在 10 mL 小瓶中

[0195] 所有三种 rPA 冻干制剂经设计以使大多数 rPA 蛋白结合于明矾,而溶液中有极少或无游离 rPA 蛋白。将 2mL 液体悬浮液装到 10mL 玻璃小瓶中。使用 FTS LyoStar® II,用以下处理参数进行冻干。

[0196] 初始冷冻:

[0197]

步骤	方法	设定
冷冻	冷冻(℃)	-60
	额外冷冻时间(分钟)	0
	冷凝器(℃)	-80
	真空(毫托)	90

[0198] 干燥:

[0199]

步骤	温度℃	时间(分钟)	R/H	真空毫托
1	-60	120	H	90
2	-28	60	R	
3	-28	1250	H	
4	-28	550	H	

[0200]

5	25	480	R
6	25	600	H
7	30	120	R
8	30	300	H
9	35	120	R
10	35	300	H
11	40	120	R
12	40	300	H
13	45	120	R
14	45	255	H

[0201] 后干：

[0202]

二级干燥设定点温度	+65℃
后加热设定	
温度(℃)	+25
时间(分钟)	1250
真空(毫托)	1250

[0203] 在接种和测试之前，所有三种制剂都用注射用水 (WFI) 复原冻干样本以产生 0.15mg/mL rPA 和 1.5mg/mL 明矾的最终浓度。通过将 1.55mL、6.2mL 和 6.18mL WFI 分别添加到第一批 (LyoA)、第二批 (LyoB) 和第三批 (LyoC) 的小瓶中来实现所述最终浓度。LyoA 的每个小瓶的最终悬浮液体积是 2.0mL 并且批次 LyoB 和 LyoC 都是 6.7mL。表 10 中示出 rPA 疫苗在复原之后的浓度。

[0204] 表 10. 冻干 rPA 疫苗在复原之后的浓度

[0205]

批号	rPA (mg/mL)	明矾 (mg/mL)	糖	氨基酸	TWEEN 80	NaCl	缓冲液	复原 WFI 体积，mL	最终体积，mL	剂量/小瓶 (0.5 mL/剂)
Lyo A	0.15	1.5	20% 海藻糖	2%丙氨酸	0.025%	-	5 mM NaPi, pH 7.0	1.55	2.0	4.0
Lyo B	0.15	1.5	6% 海藻糖	0.6%丙氨酸	0.075%	-	1.5 mM NaPi, pH 7.0	6.2	6.7	13.3

[0206]

Lyo C	0.15	1.5	6%海藻糖+0.3%蔗糖	0.6%甘氨酸	0.075%	-	6 mM Tris, pH 7.4	6.18	6.7	13.3

[0207] 在批次 LyoB 和 LyoC 中，每个小瓶的剂量总数高于批次 LyoA 的剂量总数 (13.3 相对于 4.0)。较高剂量的小瓶 (如每个小瓶 13.3 剂) 的制造成本显著低于较低剂量的小瓶 (例如每个小瓶 4 剂)。因此，开发用于制造较高剂量的小瓶的制剂和方法是一种经济的优选项。

[0208] 稳定性和测试分析

[0209] 将 rPA 液体制剂 (F1) 放到处于 5℃、25℃和 40℃的存放温度的长期稳定性程序中。将三个 rPA 冻干批次 (LyoA、LyoB 和 LyoC) 放到处于 5℃、25℃、40℃和 50℃的存放温度的长期稳定性程序中。对液体 (F1) 和冻干 rPA 疫苗制剂都进行生理化学测试。收集样本四 (4) 个月的稳定性数据。

[0210] 通过一系列分析，即 MLA、SEC-HPLC 和 AEX-HPLC 来评价 rPA 测试疫苗的生理化学性质。使用从明矾提取的 rPA 蛋白进行所有这些分析。提取程序利用 200nM 磷酸钾 /0.01％ Tw80/0.9％ NaCl。

[0211] 将存放在 -80℃下的 rPA 原料药物质 (BDS) 用作参考对照物。BDS 对照物从炭疽杆菌的 ΔSterne-1(pPA102)CR4 菌株纯化得到，所述菌株是由美国陆军传染病医学研究所 (U.S.Army Medical Research Institute of Infectious Diseases ;USAMRIID) 开发作为用于制造 rPA 的不产孢子的非产毒性表达系统。纯化 rPA BDS 并且将其在 -80℃冷冻条件下存放在 20mM Tris、0.9％ NaCl(pH 7) 中。

[0212] I. 巨噬细胞溶解分析 (MLA)

[0213] 使用体外巨噬细胞溶解分析 (MLA) 以测定 rPA 对鼠类巨噬细胞细胞系 J7774A.1 的细胞毒性。MLA 测量了 rPA 和 rLF 毒素的活性。所述分析涉及将 rPA 蛋白添加到致死因子蛋白 (rLF) 中以形成致死毒素复合物，所述复合物导致巨噬细胞的细胞膜形成孔，从而引起细胞溶解。

[0214] 相对于 BDS 参考标准测量 rPA 冻干疫苗 (使用从明矾解吸附的 rPA) 的活性，并且报告为参考标准的百分比。测定细胞存活毒素攻击的百分比。例如，rPA 疫苗的 100％ MLA 活性表示吸附于明矾并且在冻干之后的 rPA 没有损失细胞毒性活性。简言之，将小噬细胞以 5×10^4 个细胞 / 孔接种在 96 孔板中并且将板放到 CO_2 培育箱中过夜。次日，将 100ul 连续稀释的 rPA 测试样本或 rPA 参考标准 (起始 rPA 浓度为 800ng/ml，接着经 1 ： 2 稀释降至 0.8ng/mL) 与 rLF(rLF 浓度恒定在 100ng/ml) 混合并且添加到孔中。四小时之后，将 25ul 5mg/mL MTT(3-(4,5- 二甲基噻唑 2- 基)-2,5- 二苯基四唑鎓溴化物) 添加到每一孔中并且将板培育 1.5 小时。在培育 1.5 小时之后，将 100ul 可溶性溶液 (含 20％ SDS 的 50％二甲基甲酰胺溶液) 添加到孔中并且在 37℃下培育所述板过夜。次日，通过读板仪在 570nm 下读取所述板并且使用软件 (SoftMax) 用 4 参数模型将 OD 读数作图。接着将测试样本 rPA 的 ED50 值与 rPA 参考标准的 ED50 值相比，并且使用其比率来报告 rPA 测试样本的相对活

性。分析精度确定为 +/-30％。

[0215] II. 尺寸排阻色谱 (SEC-HPLC)

[0216] SEC-HPLC 是一种用于基于蛋白质分子量和尺寸来分离蛋白质的色谱技术并且其常用于评估制剂中蛋白质的稳定性。较大的蛋白质典型地比较小的蛋白质在 SEC 柱中具有更短的停留时间（更快地洗脱出）。降解（或破碎）的蛋白质的尺寸将变得较小并较迟些从色谱洗脱出。反过来也如此，聚集的蛋白质将更快地洗脱。例如，尺寸增大的聚集的 rPA 蛋白的停留时间短于天然 rPA 蛋白，并且降解（在尺寸上分解）的 rPA 蛋白将具有更长的停留时间。

[0217] SEC-HPLC 是一种常用于评估制剂中蛋白质的生理化学稳定性的分析。与 MLA 相比，SEC-HPLC 一般来说相对更敏感并且更定量，在同一运行中，其在峰面积％方面的精度小于 5％并且在停留时间方面的精度小于 0.1％。

[0218] 使用流动相为 50mM 磷酸钠 /250mM 氯化钾 (pH 7.4) 的 TSK G3000SWXL 柱 (Tosoh BioScience P/N M1182-05M) 进行 SEC-HPLC。将在 215nm 下的紫外线 (UV) 检测器或在 280nm（激发）和 335nm（发射）下的荧光检测器用于检测。

[0219] III. 阴离子交换色谱 (AEX-HPLC)

[0220] AEX-HPLC 基于蛋白质的净静电电荷来分离蛋白质。一般来说，所述分析涉及将 rPA 蛋白溶液注入配备有阴离子交换柱的 HPLC。由于静电相互作用，因此阴离子交换 (AEX) 柱（固定相）会截留 rPA 蛋白。接着通过使用离子强度 (NaCl 浓度）逐渐增大的流动相洗脱出 rPA 蛋白。rPA 分子洗脱所在的离子强度（或洗脱时间）与净电荷有关。

[0221] 已知 rPA 分子对借助于脱酰胺机制的降解敏感 (D′ Souza，Journal of Pharmaceutical Sciences，102(2)：454-461，2013)，从而引起净电荷变化。由于天门酰胺残基转化成天门冬氨酸（更负），因此脱酰胺会增加 rPA 蛋白的负电荷。与天然 rPA 蛋白相比，脱酰胺的 rPA（典型地称为酸性物质）会在更高离子强度的溶液中洗脱并且洗脱时间更长。通过主峰％表征 rPA 的纯度。

[0222] 使用 Hamilton PRP-X500 阴离子交换柱 (P/N 79641)，使用 25mM Tris(pH 8.0) 流动相 A 和 25mM Tris、0.5M NaCl(pH 8.0) 的流动相 B 进行 AEX-HPLC。类似于 SEC-HPLC，使用 UV 或荧光检测器。

[0223] IV. 免疫原性测试

[0224] 使用毒素中和分析 (TNA)(Hering 等，Biologicals 32(2004)17-27；Omland 等，Clinical and Vaccine Immunology(2008)946-953；和 Li 等，Journal of Immunological Methods(2008)333：89-106) 来评价免疫原性。将小鼠参考血清（如上所述制备）的 ED50 值用于测定测试血清样本和阳性对照物的 NF50 值。本文报告了第一个月的稳定性数据。

[0225] 对于体内免疫原性测试，使每组 20 只雌性 CD-1 小鼠接受 LyoA、LyoB、LyoC 或液体 rPA(F1) 的单次 0.5mL 腹膜内 (i.p.) 注射。评价四个稀释度剂量水平 (1 ： 4、1 ： 8、1 ： 16 和 1 ： 32)。在用盐溶液接种当天制备稀释物。在接种后第 28 天通过心脏放血来获得血清样本。

[0226] 生理化学稳定性结果

[0227] 表 11 概括了三个在 5℃、25℃、40℃和 50℃下存放 4 个月的冻干制剂和 BDS 参考对照物的 MLA、SEC-HPLC 和 AEX-HPLC 结果。

[0228] 表 11.4 个月时生理化学稳定性数据的概括

[0229]

4个月时的生理化学稳定性数据				
名称	温度,℃	MLA 相对于参考的%	SEC 纯度%,主峰	AEX 纯度%,主峰
参考BDS	-80	100	84.0	80.8
lyoA	5	116	85.8	87.1
	25	102	85.5	86.6
	40	107	83.1	84.7
lyoB	5	111	84.9	87.8
	25	112	84.9	86.2
	50	108	84.5	85.3
lyoC	5	117	84.7	87.5
	25	123	84.8	84.5
	50	104	84.7	70.5
参考BDS	-80	100	98.6	78.2
液体 rPA F1, 1个月	5	98	95.3	65.5
	25	65	91.4	25.6
	40	0	0	0

[0230] 巨噬细胞溶解分析结果

[0231] 这些 MLA 结果示出，当与 BDS 参考对照物相比时，三个在高达 40℃和 50℃的温度下存放 4 个月的冻干制剂没有显著的 rPA 细胞毒性下降（在 +/-30％的误差可变度内）。

[0232] 比较来说，在 5℃、25℃和 40℃下存放一个月的液体 rPA 疫苗 (F1) 有显著的 MLA 值下降。发现 MLA 值在 5℃、25℃和 40℃下分别是 98％、65％和 0％。图 14 示出存放 4 个月的三个冻干批次相对于存放 1 个月的 rPA 液体批次 (F1) 随存放温度变化的 MLA％的比较。这些结果示出三个冻干制剂将 rPA 疫苗的 MLA 活性在 40℃和 50℃下维持至少多达 4 个月，而液体 rPA 疫苗在 40℃下存放 1 个月之后损失所有其 MLA 活性。

[0233] 尺寸排阻色谱 (SEC-HPLC) 结果

[0234] 图 15B 中示出 rPA 参考对照物 (BDS) 的典型 SEC 层析。计算每一样本峰的峰面积百分比。天然 rPA 蛋白（单体）在 17.1 分钟洗脱，主峰面积％为 85％。第二峰在 18.2 分钟洗脱并且对应于约 15％的聚集的 rPA 分子主峰面积。通过 17.1 分钟洗脱峰的面积％确定 rPA 蛋白的纯度％，即 rPA 蛋白的纯度 (rPA 纯度％）＝ rPA 峰（对应于停留时间为 17.1 分钟的单体 rPA) 的峰面积 / 总峰面积百分比。

[0235] LyoA、LyoB 和 LyoC 的纯度结果与存放在 -80℃下的 rPA 参考 BDS 的纯度结果 84.0％相当，参见表 11。SEC-HPLC 数据示出与参考对照物相比，所有三个在所有存放温度下存放 4 个月的冻干制剂的纯度都没有显著下降。

[0236] 作为比较，当在加速温度 (25℃、40℃或 50℃）下存放 1 个月时，液体 rPA 疫苗的纯度有显著下降。液体制剂的 rPA 的相对纯度％在 5℃、25℃和 40℃下分别下降 -3.3％、-7.2％和 -98.6％。

[0237] 发现对于冻干和液体分析，BDS 参考对照物的纯度％分别是 84.0％和 98.6％。在不同时间进行两个测试。参考对照物的纯度％有差异并非不常见。所述差异可归因于变化的 SEC 柱条件和样本制备程序。典型地将相对纯度％（相对于参考对照物）用于比较在不

同时间和不同实验室的稳定性样本。

[0238] 图 15A 示出与液体制剂相比，三个冻干制剂的 rPA SEC 纯度％（相对于 BDS 参考对照物）随存放温度变化的相对降低。数据显示，在 40℃或 50℃下存放至少 4 个月的冻干 rPA 疫苗的 SEC 纯度没有变化，而液体 rPA 疫苗在 40℃下存放 1 个月之后完全降解。

[0239] 阴离子交换色谱 (AEX-HPLC) 结果

[0240] 图 16B 中示出 rPA 参考对照物 (BDS) 的典型 AEX 层析。通过主峰面积％表征 rPA 的纯度。天然 rPA 蛋白在 21.0 分钟洗脱，主峰为约 81.4％。脱酰胺 rPA（典型地称为酸性物质）在 21.7 分钟和 22.4 分钟洗脱，面积为 16.6％。通过首先将所有峰（除缓冲液峰以外）的曲线下面积积分来计算主峰面积％，然后主峰面积％对应于停留时间为 21.0 分钟的 rPA 峰（即，主峰面积％＝峰面积 (RT ＝ 21.0 分钟）/ 所有峰面积的总和）。类似于 SEC-HPLC，rPA 纯度与主峰面积％相同。AEX 分析的精度为约 10-15％。

[0241] 发现存放 4 个月的第一批冻干物 (lyoA) 的 AEX 纯度％在 5℃、25℃和 40℃下分别为 87.1、86.6、84.7。第二批冻干物 (lyoB) 的 AEX 纯度在 5℃、25℃和 50℃下分别为 87.8％、86.2％和 85.3％。第三批冻干物 (lyoC) 的 AEX 纯度％在 5℃、25℃和 50℃下分别为 87.5％、84.5％和 70.5％。LyoA、LyoB 和 LyoC 的这些纯度结果与 rPA 参考对照物 (BDS) 的纯度 80.8％相当。所有三个在所有存放温度下存放 4 个月后的冻干制剂的纯度相对于参考对照物都没有显著下降。

[0242] 发现存放 1 个月的液体 rPA 疫苗的 AEX 纯度在 5℃、25℃和 40℃下分别为 65.5％、25.6％和 0％；并且当用于分析测试液体 rPA 时，rPA 参考对照物 (BDS) 的 AEX 纯度为 78.2％。将 AEX 纯度结果概述于表 11 中。

[0243] 如图 16A 中所示，与冻干制剂相比，在 5℃、25℃和 40℃下的 rPA 液体制剂 (F1) 中的 AEX 纯度有显著下降。AEX 数据显示，在 40℃或 50℃下存放至少 4 个月的冻干 rPA 疫苗的纯度没有损失；而液体 rPA 疫苗在 40℃下存放 1 个月之后完全降解。

[0244] 体内免疫原性结果

[0245] 测定三个存放 1 个月的冻干制剂各自在小鼠中在剂量水平 0.25、0.125、0.0625 和 0.03125 下的 NF50 结果。图 17A-D、18A-D 和 19A-D 比较了在多个温度 (5℃、25℃和 40℃）下在其相应制剂和剂量水平下的 NF50 数据 (n ＝ 20)。

[0246] 表 12A-C 中概括了三个冻干制剂的四个剂量水平在 5℃、25℃和 40℃下的 NF50 的几何平均数 (<NF50>gm)。

[0247] 表 12A-C. 三个冻干制剂在 5℃、25℃和 40℃下在四个剂量水平 (0.25、0.125、0.0625 和 0.03125) 下的 NF50 的几何平均值 (n ＝ 20)

[0248]

13A. 在四个剂量水平下在 5℃、25℃、和 40℃下存放一个月的 Lyo A 的<NF50> gm

剂量水平	5 ℃	25 ℃	40 ℃	在各温度下的<NF> gm 显著不同吗？(p<0.050) ANOVA 检验
0.25	1.12	1.20	1.13	不，p = 0.97
0.125	0.84	0.86	0.50	不，p = 0.10
0.0625	0.33	0.28	0.39	不，p = 0.36
0.03125	0.10	0.15	0.13	不，p = 0.18

[0249]

13B. 在四个剂量水平下在 5℃、25℃、40℃和50℃下存放一个月的 Lyo B 的<NF50> gm

剂量水平	5 ℃	25 ℃	40 ℃	在各温度下的<NF> gm 显著不同吗？(p<0.050) ANOVA 检验
0.25	1.16	0.81	0.86	不，p = 0.22
0.125	0.33	0.46	0.32	不，p = 0.20
0.0625	0.12	0.15	0.13	不，p = 0.59
0.03125	0.05	0.06	0.05	不，p = 0.78

[0250]

13C. 在四个剂量水平下在 5℃、25℃、40℃和50℃下存放一个月的31 Lyo C 的<NF50> gm

剂量水平	5 ℃	25 ℃	40 ℃	在各温度下的<NF> gm 显著不同吗？(p<0.050) ANOVA 检验
0.25	0.98	0.80	1.02	不，p = 0.37
0.125	0.43	0.45	0.46	不，p = 0.94
0.0625	0.13	0.15	0.20	不，p = 0.21
0.03125	0.07	0.06	0.07	不，p = 0.78

[0251] 对于存放 1 个月之后的批次 LyoA，发现在剂量水平 0.25 下在 5℃、25℃和 40℃下的 <NF50>gm 分别是 1.12、1.20 和 1.13。在三个存放温度 (5℃、25℃和 40℃) 下，<NF50>gm 没有统计上显著的差异，p = 0.97。类似地，还示出在多个存放温度下在其它测试剂量水平 (0.125、0.0625 和 0.01315) 下，所有三个冻干制剂 (LyoA、LyoB 和 LyoC) 没有统计差异。NF50 数据显示在高达 40℃下 1 个月的三个冻干制剂的免疫原性没有显著下降。

[0252] 相反地，在 25℃和 40℃存放温度下 1 个月，液体 rPA 制剂 (F1) 示出显著的免疫原性下降。表 13 示出在 5℃、25℃和 40℃下存放 1 个月的 rPA 液体制剂的 <NF50>gm 结果。

[0253] 表 13. 在四个剂量水平 (0.3、0.2、0.1 和 0.05) 下在 5℃、25℃和 40℃下存放 1 个月的液体 rPA 制剂的 NF50 的几何平均值

[0254]

在四个剂量水平下在5℃、25℃、和40℃下存放一个月的液体rPA制剂(F1)的<NF50> gm				
剂量水平	5 ℃	25 ℃	40 ℃	在各温度下的<NF> gm 显著不同吗?(p<0.050) ANOVA检验
0.3	1.26	0.69	0.30	是, p = 0.0023
0.2	1.18	0.55	0.13	是, p = <0.0001
0.1	1.03	0.22	0.09	是, p = <0.0001
0.05	0.32	0.15	0.03	是, p = 0.0010

[0255] 在0.3剂量水平下,发现在5℃、25℃和40℃下的<NF50>gm分别是1.26、0.69和0.30,并且所有<NF50>gm都有统计上显著的差异,p = 0.0023。类似地,在较低的剂量水平0.2、0.1和0.05下,<NF50>gm随着存放温度提高而显著下降(参见图20A-D)。发现当将液体疫苗存放在25℃下,如相比于5℃下时,在所有四个剂量水平下,NF50都显著减小,并且在40℃下会逐渐增大。

[0256] 总之,免疫原性数据显示冻干的rPA制剂优越于液体rPA制剂。冻干制剂在25℃和40℃下维持其免疫原性至少1个月,而液体制剂的免疫原性在类似的存放条件下会显著降低。

[0257] 像大多数液体疫苗那样,发现液体rPA疫苗在加速存放温度(例如25℃和40℃)下不稳定。液体rPA疫苗当在40℃下存放1个月时会损失其免疫原性和关键生理化学性质。类似地,疫苗的关键生理化学性质还会显著降级。发现如通过巨噬细胞溶解分析(MLA)、尺寸排阻色谱(SEC-HPLC)和阴离子交换色谱(AEX-HPLC)测量的疫苗含量和纯度在25℃下会显著降低并且在40℃下1个月会不可检测。已知液体rPA疫苗对脱酰胺反应敏感,尤其是当其吸附于铝上并且存放在加速温度下时。

[0258] 制造以下批号的三个冻干制剂:lyoA、lyoB和lyoC。这三个新的rPA冻干制剂比rPA液体疫苗具有优越的稳定性概况。对于所有三批冻干制剂,当在高达50℃的温度下存放4个月时,所有关键生理化学分析(MLA、SEC-HPLC和AEX-HPLC)中的纯度都没有统计上显著的变化。另外,当在四个剂量水平下在高达40℃下存放冻干疫苗1个月时,免疫原性(NF50)没有显著的下降。

[0259] 本文的结果示出冻干的rPA制剂比液体rPA制剂具有优越的生理化学和免疫学稳定性概况。在高达50℃的存放温度下并且历经4个月的存储时间,冻干制剂维持所有通过MLA、SEC和AEX测试的关键生理化学性质。在高达40℃的存放温度下至少1个月,冻干制剂还维持免疫原性。相反地,在40℃下存放1个月之后,液体rPA制剂示出完全失去生理化学性质(MLA、SEC和AEX)并且免疫原性显著下降。

[0260] 实施例7:用其它佐剂的制剂和冻干

[0261] 将CPG 7909原料药物质(BDS)以冻干粉形式包装于高密度聚乙烯(HDPE)瓶中,热密封于多层(聚酯薄膜,箔)囊中,并且存放在-20℃ ±5℃下。

[0262] 从Avanti Polar Lipids,Inc获得葡萄哌喃糖基脂质佐剂(GLA)。将其包装于含有25mg冻干GLA粉末的2mL琥珀色玻璃小瓶中并且存放在-20℃ ±5℃下。Arias等.(2012)PLoS ONE 7(7):e41144中描述了GLA。

[0263] 使用含原料药物质(BDS):2.81mg/mL rPA、0.9%NaCl且缓冲于20mM Tris-HCl中

(pH 7.4)。使用前将其存放在 -80℃下并且在 5℃下解冻过夜。

[0264] 从 InviviGen,在 20mL 玻璃小瓶中以含有 50mg PIPC 的冻干饼形式获得 PolyI PolyC(PIPC)。将其存放在 5℃下。

[0265] 表 14. 化学品和来源

[0266]

化学名称	来源
超纯 Tris 盐酸盐	Amresco
2% ALHYDROGEL(10mg/mL 铝)	Brenntag
二水合 α,α-海藻糖	Hayashibara Biochemicals
无水磷酸二氢钠	Sigma Aldrich
七水合磷酸氢二钠	BDH
聚山梨酯 80,N.F.	J.T.Baker
L- 丙氨酸	EMD

[0267] 表 15. 设备 / 材料

[0268]

名称	来源
VirTis Advantage Plus 冻干器	SP Scientific
Wheaton 血清小瓶,硼硅酸盐玻璃	VWR
Lyo 小瓶用开槽橡皮塞	VWR
翻盖型压接封盖	VWR

[0269] 储备溶液制备物

[0270] 分别在 20mM Tris 和 5mM NaPi 缓冲液中制备两种 60% (w/v) 海藻糖溶液,并且将其无菌过滤。在 DI 水中制备 10% (v/v) 聚山梨醇酯 80 溶液,接着无菌过滤。分别在 20mM Tris 和 5mM NaPi 缓冲液中制备两种 12% (w/v) 丙氨酸溶液,并且将其无菌过滤。

[0271] 通过将 7mL 1M Tris 缓冲液 (pH 7.4) 添加到 343mL 2% AlOH(或 10mg/mL 铝) 中来缓冲一个氢氧化铝储备溶液并且滴定至 pH 7.4。通过将 1.75mL 1M NaPi 缓冲液 (pH 7.0) 添加到 348.25mL 2% AlOH 中来缓冲第二个氢氧化铝储备溶液并且滴定至 pH 7.0。在任一制备物中都不考虑添加缓冲液和后续滴定所产生的稀释效应。

[0272] 佐剂制备物

[0273] 通过将 31.2mg 粉末添加到 50mL 锥形管中并添加 15.6mL 缓冲液而在 20mM Tris-HCl 缓冲液 (pH 7.4) 中制备 GLA 佐剂。在 10 秒的间隔内 (其间其余部分为 10 秒,至

最大功率 15W) 将混合物超声处理总共 60 秒。获得 2mg/mL GLA 的混浊混合物。

[0274] 在 20mM Tris(pH 7.4) 或 5mM NaPi(pH 7.0) 缓冲液中制备 CPG 7909 备料。在每一制备物中,将约 200mg CPG 7909 粉末完全溶解于 10mL 的最终体积中。两种制备物都获得透明溶液。

[0275] 通过将 50mg PIPC 冻干饼溶解在 25mL 5mM NaPi 缓冲液 (pH7.0) 中来制备 2mg/mL PIPC 储备溶液。在 10 秒的间隔内 (其间其余部分为 10 秒,至最大功率 15W) 将混合物超声处理总共 60 秒。没有获得具有可见粒子的透明溶液。

[0276] 共混用配制程序

[0277] 表 16 示出所制备的制剂的化学组成 :

[0278] 表 16 :液体和冻干的制剂共混物

[0279]

样本号	研究组	化学组成										
		rPA (mg/mL)	明矾 (mg/mL)	海藻糖 (%)	Tween 80 (%)	佐剂 (mg/mL)	丙氨酸 (%)	Tris-HCl 缓冲液 (mM)	NaPi 缓冲液 (mM)	pH	佐剂名称	NaCl (残留) (mM)
7-13	CPG-液体	0.15	1.5	0	0.03	0.50	0	20	0	7.4	CPG	8
14-20	CPG-Lyo	0.15	1.5	20	0.03	0.50	2	20	0	7.4	CPG	8
34-39	GLA-液体	0.15	1.5	0	0.03	0.50	0	20	0	7.4	GLA	8
40-45	GLA-Lyo	0.15	1.5	20	0.03	0.50	2	20	0	7.4	GLA	8
73-75	PIPC/CPG-液体	0.15	1.5	0	0.03	0.50	0	1.1	5	7.0	PIPC+CPG	8
76-81	PIPC/CPG-LYO	0.15	1.5	20	0.03	0.50	2	1.1	5	7.0	PIPC+CPG	8

[0280] 使用如表 17 中所示的缓冲储备溶液制备表 16 中的制剂共混物。

[0281] 表 17 :用于制剂共混物制备物的备料 / 赋形剂体积

[0282]

研究组	添加的制剂体积(mL)储备溶液										最终体积 (mL)
	rPA 备料 (mg/mL)	AlOH 备料 (mg/mL)	海藻糖 备料 (%)	Tween 80 备料 (%)	丙氨酸 备料 (%)	CpG 备料 (mg/mL)	GLA 备料 (mg/mL)	Poly(IC) 备料 (mg/mL)	NaPi 缓冲液 (mM)	Tris 缓冲液 (mM)	
	2.8	10	60	10	12	20	2	2	5	20	
CPG-液体	0.86	2.40	0	0.040	0	0.40	0	0	0	12.30	16
CPG-Lyo	1.07	3.00	6.67	0.050	3.333	0.50	0	0	0	5.38	20
GLA-液体	0.86	2.40	0	0.040	0	0	4.0	0	0	8.70	16
GLA-Lyo	1.07	3.00	6.67	0.050	3.333	-	5.0	0	0	0.88	20
PIPC/CPG-液体	0.86	2.40	0	0.040	0	0.40	0	4.0	8.30	0	16
PIPC/CPG-LYO	1.07	3.00	6.67	0.050	3.333	0.50	0	5.0	0.38	0	20

[0283] 用于共混所有赋形剂的添加顺序如下 :氢氧化铝→海藻糖→ TWEEN 80 →丙氨酸→缓冲液→ rPA → CPG 或 GLA 佐剂

[0284] 为样本 14-20、40-45 和 76-81 制备 20mL 的最终体积以便冻干。将共混物以 2mL 等分试样分到 10mL 玻璃小瓶中并且放在一旁以便冻干。

[0285] 冻干程序

[0286] 使用 VirTis Plus冷冻干燥器冻干样本。如表 18 中所述采用和输入 73 小时循环。

[0287] 表 18 :冻干循环

[0288]

编号	步骤	温度(°C)	时间(小时)	勻变/保持	真空(毫托)
1	热处理	-60	快速	-	-
2		-60	2	H	
3		-28	1	R	
4		-28	20	H	
5	额外冷冻	-28	10	H	20
6	初级干燥	25	8	R	20
7		25	10	H	
8		30	1	R	
9		30	5	H	
10	二级干燥	35	1	R	
11		35	5	H	
12		40	1	R	
13		40	4	H	
14		45	1	R	
15		45	4	H	
后干燥		25	-	-	3000

[0289] 数据概括

[0290] 在使用腹膜内途径免疫一次的小鼠 (n = 10) 中测试六个疫苗制剂的免疫学反应。在免疫之后第 28 天收集血清。如实施例 3 中所述测定在剂量水平 = 0.4 下的 TNA 数据并且结果概括于表 19 中。

[0291] 发现 CPG- 液体、CPG-lyo、GLA- 液体、GLA-lyo、PIPC/CPG- 液体和 PIPC/CPG-lyo 样本的平均 NF50 分别是 53.6、48.9、69.9、64.7、89.4 和 77.3。CPG、GLA 和 PIPC/CPG 的液体相对于冻干制剂的平均 NF50 没有统计差异。数据显示冻干制剂和方法甚至在其它佐剂存在下也能维持免疫原性。

[0292] 表 19 :含有 CPG、GLA 和 PIPC/CPG 佐剂的疫苗的液体相对于冻干制剂的 NF50

[0293]

n	NF50 (DL =0.4)					
	CPG-液体	CPG-Lyo	GLA-液体	GLA-Lyo	PIPC/CPG-液体	PIPC/CPG-液体
1	51.2	43.1	114.4	108.9	91.6	62.8
2	106.3	29.2	115.2	23.7	100.8	67.2
3	103.9	77.4	43.1	65.1	114.5	119.2
4	33.1	17.2	50.3	60.9	74.6	84.0
5	10.3	47.8	122.0	83.3	19.4	63.2
6	37.3	31.4	55.9	29.2	249.5	68.6
7	47.6	51.8	50.1	115.8	61.0	84.8
8	54.7	66.4	33.3	79.3	65.3	62.4
9	42.8	36.5	81.7	67.9	29.8	79.4
10	48.9	87.9	32.5	12.9	87.4	81.2
平均值	53.6	48.9	69.9	64.7	89.4	77.3
标准差	29.9	22.5	35.5	34.6	63.6	17.3
P值 T-检验	0.695		0.746		0.573	

[0294]

n	Log NF50 (DL=0.4)					
	CPG-液体	CPG-Lyo	GLA-液体	GLA-Lyo	PIPC/CPG-液体	PIPC/CPG-液体
1	1.7	1.6	2.1	2.0	2.0	1.8
2	2.0	1.5	2.1	1.4	2.0	1.8
3	2.0	1.9	1.6	1.8	2.1	2.1
4	1.5	1.2	1.7	1.8	1.9	1.9
5	1.0	1.7	2.1	1.9	1.3	1.8
6	1.6	1.5	1.7	1.5	2.4	1.8
7	1.7	1.7	1.7	2.1	1.8	1.9
8	1.7	1.8	1.5	1.9	1.8	1.8
9	1.6	1.6	1.9	1.8	1.5	1.9
10	1.7	1.9	1.5	1.1	1.9	1.9
平均值对数	1.7	1.6	1.8	1.7	1.9	1.9
几何平均值	45.6	44.1	62.2	53.7	72.4	75.8
标准差	0.3	0.2	0.2	0.3	0.3	0.1
P值 T-检验	0.898		0.607		0.849	

[0295] 实施例 8 :冻干 rPA 疫苗的免疫原性

[0296] 本实施例比较了新鲜制备的液体疫苗相对于在 5℃和 50℃下存放 1 个月的冻干疫苗,并且比较了在 NaPi(pH 7.0) 相对于柠檬酸 (pH5.5) 缓冲液中制备的 CPG 制剂。

[0297] 在本研究下评价四个制剂 :

[0298] 含 rPA 明矾的 NaPi(pH 7.0)

[0299] 含 rPA 明矾 +CPG 的 NaPi(pH 7.0)

[0300] 含 rPA 明矾 +CPG 的柠檬酸 (pH 5.5)

[0301] 含 rPA 明矾 +GLA 的 Tris(pH 7.4)

[0302] 储备溶液制备物

[0303] 制备三个 60% (w/v) 海藻糖溶液,一个在 20mM Tris(pH 7.4) 中,第二个在 5mM NaPi 缓冲液 (pH 7.0) 中,并且第三个在 20mM 中柠檬酸钠 (pH 5.5) 中。通过在 90mL DI 水中混合 10mL 浓缩 Tween 80 来制备 10% (v/v) 聚山梨醇酯 80 溶液。制备三个 12% (w/v) 丙氨酸溶液,一个在 20mM Tris(pH 7.4) 中,第二个在 5mM NaPi 缓冲液 (pH 7.0) 中,并且第三个在 20mM 中柠檬酸钠 (pH 5.5) 中,并且所有都被无菌过滤。

[0304] 通过将 1M 缓冲液添加到 2% AlOH 中来缓冲三个氢氧化铝储备溶液。将所得备料滴定至所需 pH 值以在使用时匹配缓冲液。在任何制备物中都不考虑添加缓冲液和后续滴定所产生的稀释效应。

[0305] 表 19 :在冻干之前的制剂

[0306]

编号	缓冲液	存放条件	rPA mg/mL	铝 mg/mL	海藻糖 %	丙氨酸 %	CPG mg/mL	GLA mg/mL	TWEEN 80 (%)
1	5mM NaPI pH 7.0	新鲜液体	0.5	5.0	20.0	2.0	-	-	0.025
2		Lyo 5°C, 1 mo							
3		Lyo 50°C, 1 mo							
4	5mM NaPI pH 7.0	新鲜液体	0.45	4.5	20.0	2.0	1.5	-	0.025
5		Lyo 5°C, 1 mo							
6		Lyo 50°C, 1 mo							
7	柠檬酸钠 pH 5.5	新鲜液体	0.45	4.5	20.0	2.0	1.5	-	0.025
8		Lyo 5°C, 1 mo							
9		Lyo 50°C, 1 mo							
10	20mM Tris-HCL pH 7.4	新鲜液体	0.45	4.5	20.0	0.0	-	0.30	0.025
11		Lyo 5°C, 1 mo							
12		Lyo 50°C, 1 mo							

[0307] 共混每一配制,接着使用如实施例 7 中所述的冻干方法以每小瓶 2mL 冻干。

[0308] 表 20 :新鲜液体或复原浓聚物

[0309]

编号	缓冲液	存放条件	rPA mg/mL	铝 mg/mL	海藻糖 %	丙氨酸 %	CPG mg/mL	GLA mg/mL	TWEEN 80 (%)
1	5mM NaPI pH 7.0	新鲜液体	0.15	1.5	6.06	0.61	0	0	.008
2		Lyo 5°C, 1 mo							
3		Lyo 50°C, 1 mo							
4	5mM NaPI pH 7.0	新鲜液体	0.15	1.5	6.67	0.67	.5	0	.008
5		Lyo 5°C, 1 mo							
6		Lyo 50°C, 1 mo							
7	柠檬酸钠 pH 5.5	新鲜液体	0.15	1.5	6.67	0.67	.5	0	.008
8		Lyo 5°C, 1 mo							
9		Lyo 50°C, 1 mo							
10	20mM Tris-HCL pH 7.4	新鲜液体	0.15	1.5	6.67	0	0	0.1	.008
11		Lyo 5°C, 1 mo							
12		Lyo 50°C, 1 mo							

[0310] 动物模型

[0311] 每一动物接收 0.5mL 1/16 稀释度的所列制剂。每组 5 只雌性 /5 只雄性豚鼠 (n = 10)。在第 0 天和第 14 天进行 IM 免疫。在第 14、28 和 35 天进行采血。分析和呈现在第 28 天的 TNA 数据。

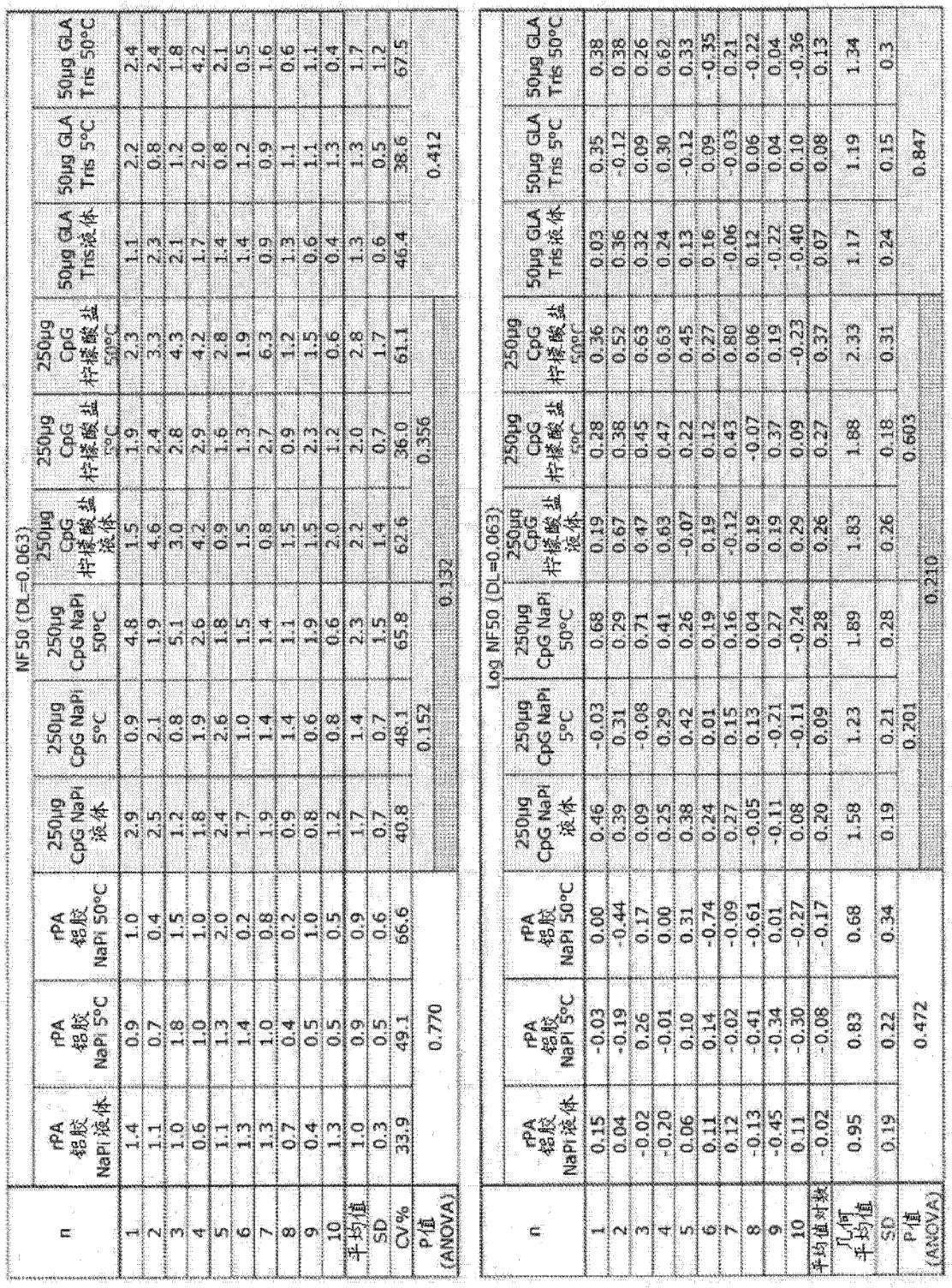
[0312] NF50 数据

[0313] 图 21 示出 12 种制剂的 NF50 和平均数标准差。

[0314] 表 21 示出 12 种制剂的每一只小鼠的数值。

[0315] 表 21 :12 种制剂中每一种的每一只小鼠的 NF50 和 LogNF50。

[0316]



NF50 (DL=0.063)

n	rPA 铝胶 NaPi 液体	rPA 铝胶 NaPi 5°C	rPA 铝胶 NaPi 50°C	250μg CpG NaPi 液体	250μg CpG NaPi 5°C	250μg CpG NaPi 50°C	250μg CpG 柠檬酸盐液体	250μg CpG 柠檬酸盐 5°C	250μg CpG 柠檬酸盐 50°C	50μg GLA Tris液体	50μg GLA Tris 5°C	50μg GLA Tris 50°C
1	1.4	0.9	1.0	2.9	0.9	4.8	1.5	1.9	2.3	1.1	2.2	2.4
2	1.1	0.7	0.4	2.5	2.1	1.9	4.6	2.4	3.3	2.3	0.8	2.4
3	1.0	1.8	1.5	1.2	0.8	5.1	3.0	2.8	4.3	2.1	1.2	1.8
4	0.6	1.0	1.0	1.8	1.9	2.6	4.2	2.9	4.2	1.7	2.0	4.2
5	1.1	1.3	2.0	2.4	2.6	1.8	0.9	1.6	2.8	1.4	0.8	2.1
6	1.3	1.4	0.2	1.7	1.0	1.5	1.5	1.3	1.9	1.4	1.2	0.5
7	1.3	1.0	0.8	1.9	1.4	1.4	0.8	2.7	6.3	0.9	0.9	1.6
8	0.7	0.4	0.2	0.9	1.4	1.1	1.5	0.9	1.2	1.3	1.1	0.6
9	0.4	0.5	1.0	0.8	0.6	1.9	1.5	2.3	1.5	0.6	1.1	1.1
10	1.3	0.5	0.5	1.2	0.8	0.6	2.0	1.2	0.6	0.4	1.3	0.4
平均值	1.0	0.9	0.9	1.7	1.4	2.3	2.2	2.0	2.8	1.3	1.3	1.7
SD	0.3	0.5	0.6	0.7	0.7	1.5	1.4	0.7	1.7	0.6	0.5	1.2
CV%	33.9	49.1	66.6	40.8	48.1	65.8	62.6	36.0	61.1	46.4	38.6	67.5
P值 (ANOVA)	0.770			0.152		0.132	0.356			0.412		

Log NF50 (DL=0.063)

n	rPA 铝胶 NaPi 液体	rPA 铝胶 NaPi 5°C	rPA 铝胶 NaPi 50°C	250μg CpG NaPi 液体	250μg CpG NaPi 5°C	250μg CpG NaPi 50°C	250μg CpG 柠檬酸盐液体	250μg CpG 柠檬酸盐 5°C	250μg CpG 柠檬酸盐 50°C	50μg GLA Tris液体	50μg GLA Tris 5°C	50μg GLA Tris 50°C
1	0.15	-0.03	0.00	0.46	-0.03	0.68	0.19	0.28	0.36	0.03	0.35	0.38
2	0.04	-0.19	-0.44	0.39	0.31	0.29	0.67	0.38	0.52	0.36	-0.12	0.38
3	-0.02	0.26	0.17	0.09	-0.08	0.71	0.47	0.45	0.63	0.32	0.09	0.26
4	-0.20	-0.01	0.00	0.25	0.29	0.41	0.63	0.47	0.63	0.24	0.30	0.62
5	0.06	0.10	0.31	0.38	0.42	0.26	-0.07	0.22	0.45	0.13	-0.12	0.33
6	0.11	0.14	-0.74	0.24	0.01	0.19	0.19	0.12	0.27	0.16	0.09	-0.35
7	0.12	-0.02	-0.09	0.27	0.15	0.16	-0.12	0.43	0.80	-0.06	-0.03	0.21
8	-0.13	-0.41	-0.61	-0.05	0.13	0.04	0.19	-0.07	0.06	0.12	0.06	-0.22
9	-0.45	-0.34	0.01	-0.11	-0.21	0.27	0.19	0.37	0.19	-0.22	0.04	0.04
10	0.11	-0.30	-0.27	0.08	-0.11	-0.24	0.29	0.09	-0.23	-0.40	0.10	-0.36
平均值对数	-0.02	-0.08	-0.17	0.20	0.09	0.28	0.26	0.27	0.37	0.07	0.08	0.13
几何平均值	0.95	0.83	0.68	1.58	1.23	1.89	1.83	1.88	2.33	1.17	1.19	1.34
SD	0.19	0.22	0.34	0.19	0.21	0.28	0.26	0.18	0.31	0.24	0.15	0.3
P值 (ANOVA)	0.472			0.201		0.210	0.603			0.847		

[0317] NF50 数据示出四个冻干制剂的优越稳定性。还显示制剂的稳健性。

[0318] 对于所有四个制剂，在新鲜液体、lyo（在 5℃和 50℃下 1 个月）之间，NF50 平均值和几何平均值没有统计差异：（参见表 21）

[0319] 对于 rPA 明矾 +CPG 制剂，在 NaPi 相对于柠檬酸缓冲液之间，NF50 的平均值和几何平均值没有统计差异。（参见表 21）

[0320] 实施例 9：含流感抗原的制剂

[0321] 使用类似于本文关于 rPA 制剂所公开的方法配制含有流感抗原的制剂，除了存在

流感抗原和不存在 rPA 抗原的情况以外。

[0322] 将含有流感抗原的制剂的实例列于表 20 中。

[0323] 表 22. 用于冻干的含有流感抗原的制剂的实例

[0324]

制剂编号	流感抗原 mg/ml	明矾 mg/ml	糖	氨基酸	TWEEN 80	液体冻干物体积
1	0.15	1.5	20%海藻糖	无	0.025%	2 mL
2	0.15	1.5	30%海藻糖	无	0.025%	2 mL
3	0.15	1.5	20%海藻糖	2% Ala	0.025%	2 mL
4	0.15	1.5	30%海藻糖	2% Ala	0.025%	2 mL
5	0.15	1.5	20%海藻糖	2% Gly	0.025%	2 mL
6	0.15	1.5	30%海藻糖	2% Gly	0.025%	2 mL
7	0.15	1.5	20%海藻糖	2% Arg	0.025%	2 mL
8	0.15	1.5	30%海藻糖	2% Arg	0.025%	2 mL
9	0.15	1.5	10%海藻糖	2% Ala	0.025%	2 mL
10	0.15	1.5	10%海藻糖	2% Gly	0.025%	2 mL
11	0.15	1.5	10%海藻糖	2% Arg	0.025%	2 mL
12	0.5	5	20%海藻糖	无	0.025%	2 mL
13	0.5	5	30%海藻糖	无	0.025%	2 mL
14	0.5	5	20%海藻糖	2% Ala	0.025%	2 mL
15	0.5	5	30%海藻糖	2% Ala	0.025%	2 mL
16	0.5	5	20%海藻糖	2% Gly	0.025%	2 mL
17	0.5	5	30%海藻糖	2% Gly	0.025%	2 mL
18	0.5	5	20%海藻糖	2% Arg	0.025%	2 mL
19	0.5	5	30%海藻糖	2% Arg	0.025%	2 mL
20	0.5	5	10%海藻糖	2% Ala	0.025%	2 mL
21	0.5	5	10%海藻糖	2% Gly	0.025%	2 mL
22	0.5	5	10%海藻糖	2% Arg	0.025%	2 mL

[0325] 制备两组制剂，一组在 5mM NaPi(pH 7.0) 缓冲液或 20mM Tris(pH 7.4) 缓冲液中。流感抗原是流感血凝素。制剂也可以含有另一佐剂如 0.5mg/mL CPG、0.5mg/mL PIPC、0.1mg/Ml GLA 或其组合。

[0326] 使用如实施例 7 中所述的冻干方法以每小瓶 2mL 冻干每一制剂。

[0327] 在免疫性研究、稳定性研究和 / 或功效研究中测试每一制剂。

[0001]

序列表

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Ruiz, Christian Fernando
Miles, Aaron Paul
Welsh, Richard William

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Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
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Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
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[0002]

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Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
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Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
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Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
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Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
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Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
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Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
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Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
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Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
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Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
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[0003]

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Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
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Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
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Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
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Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
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Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
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Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
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[0005]

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Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
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Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
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Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
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Thr Phe Ala Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp
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Gln Glu Val Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu
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Lys Gly Arg Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro
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Thr Glu Lys Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn
100 105 110

Lys Lys Glu Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys
115 120 125

Gln Lys Ser Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro
130 135 140

Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val
145 150 155 160

Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro
165 170 175

[0007]

Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser
180 185 190

Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu
195 200 205

Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His
210 215 220

Pro Leu Val Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile
225 230 235 240

Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Gln
245 250 255

Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser
260 265 270

Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly
275 280 285

Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala
290 295 300

Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr
305 310 315 320

Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg
325 330 335

Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr
340 345 350

[0008]

Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys
355 360 365

Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser
370 375 380

Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser
385 390 395 400

Thr Pro Ile Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr
405 410 415

Lys Gln Leu Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr
420 425 430

Tyr Asn Phe Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp
435 440 445

Ser Glu Val Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe
450 455 460

Asn Gly Lys Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn
465 470 475 480

Pro Ser Asp Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu
485 490 495

Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln
500 505 510

Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln
515 520 525

[0009]

Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn
530 535 540

Ile Tyr Thr Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile
545 550 555 560

Leu Ile Arg Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val
565 570 575

Gly Ala Asp Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn
580 585 590

Ser Ser Thr Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys
595 600 605

Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys
610 615 620

Glu Val Ile Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg
625 630 635 640

Gln Asp Gly Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu
645 650 655

Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val
660 665 670

Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser
675 680 685

Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu
690 695 700

[0010]

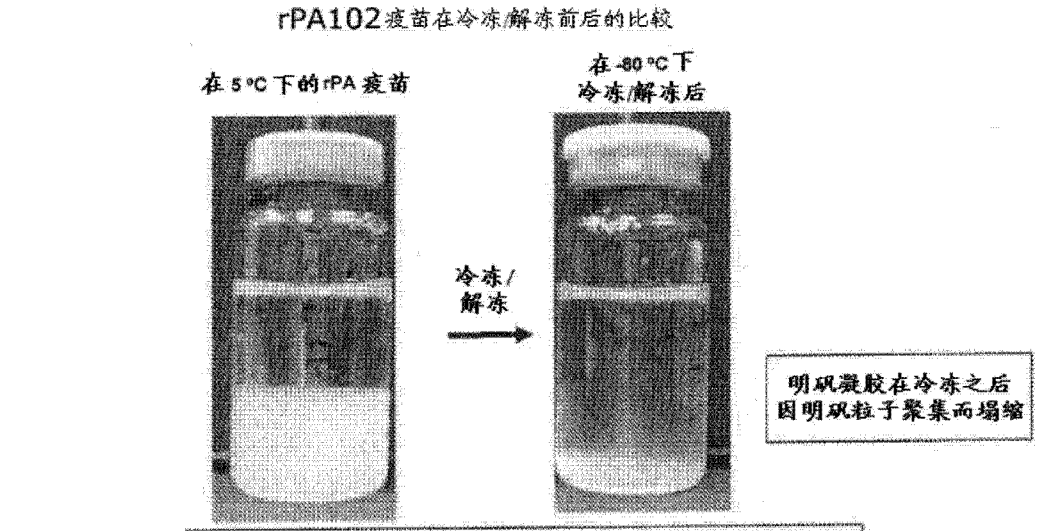
Ile Gly
705

<210> 3
<211> 4
<212> PRT
<213> 人工序列

<220>
<223> 合成多肽

<400> 3

Arg Lys Lys Arg
1



冷冻/解冻	完整rPA102		解吸附的rPA102		
	荧光 (320/350)	体内 (MRPT)**	MLA	RP-HPLC (纯度)	SE-HPLC (单体%)
冷冻前	1.21	0.38	18%	82.84	94.58
冷冻后	0.94*	0.20***	2%	74.95	79.8

*表示在冷冻之后rPA102蛋白结构的变化，数据来自AD (AD12117 FD样本)
** 数据来自MRPT组
***在95% CI下统计显著

- 冷冻破坏了rPA102疫苗
- 效力(MRPT)数据与生理化学和凝胶高度(塌缩)相关

图 1

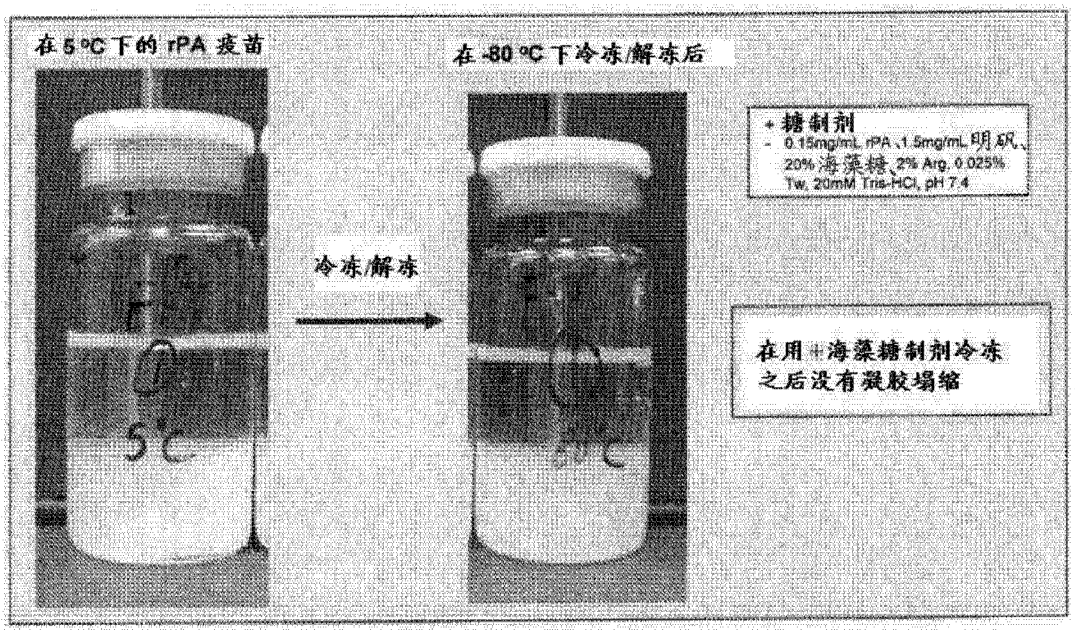

在 5 °C 下的 rPA 疫苗
在 -80 °C 下冷冻/解冻后
冷冻/解冻
5 °C
+ 糖制剂
- 0.15mg/mL rPA、1.5mg/mL 明矾、20% 海藻糖、2% Arg, 0.025% Tw, 20mM Tris-HCl, pH 7.4
在用+海藻糖制剂冷冻之后没有凝胶塌缩

图 2

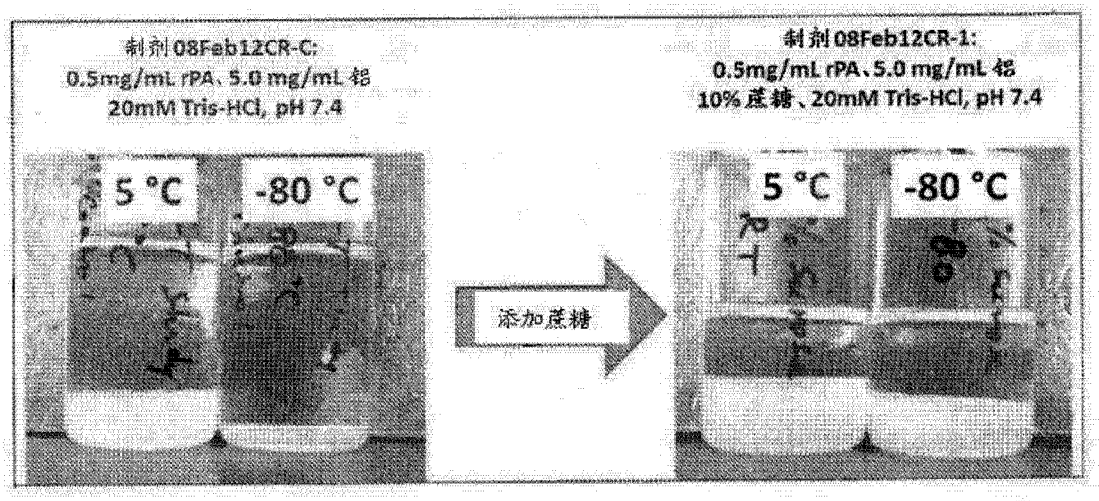

制剂 08Feb12CR-C:
0.5mg/mL rPA、5.0 mg/mL 铝
20mM Tris-HCl, pH 7.4
5 °C
-80 °C
添加蔗糖
制剂 08Feb12CR-1:
0.5mg/mL rPA、5.0 mg/mL 铝
10% 蔗糖、20mM Tris-HCl, pH 7.4
5 °C
-80 °C

图 3

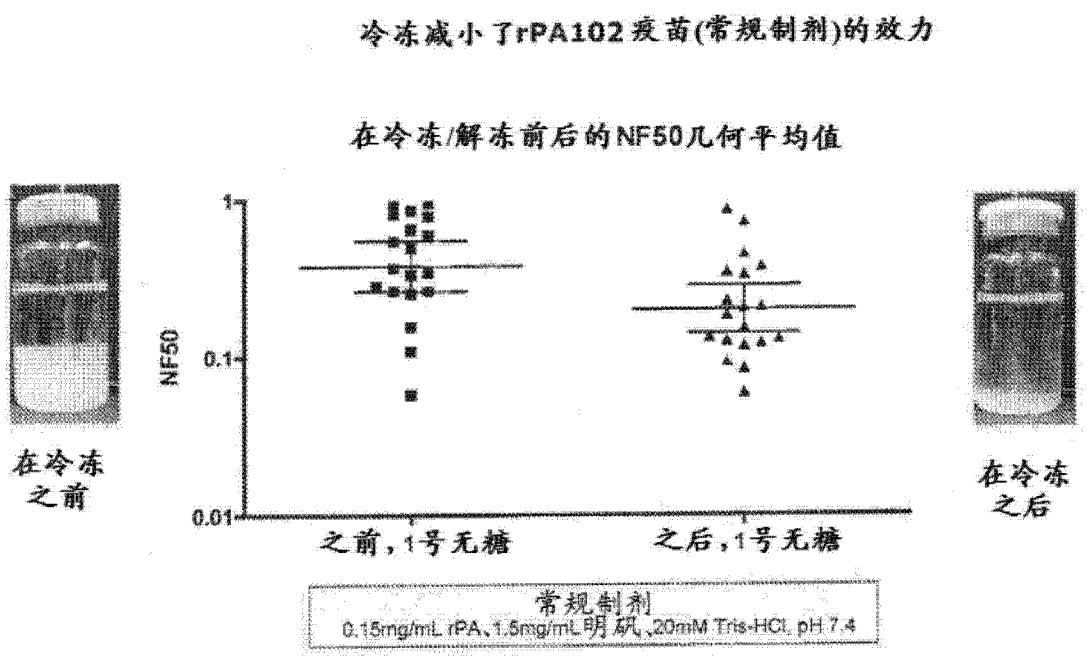

冷冻减小了rPA102疫苗(常规制剂)的效力
在冷冻/解冻前后的NF50几何平均值
在冷冻之前
NF50
1
0.1
0.01
之前，1号无糖
之后，1号无糖
在冷冻之后
常规制剂
0.15mg/mL rPA、1.5mg/mL 明矾、20mM Tris-HCl, pH 7.4
• 在冷冻之后，19只小鼠的NF50几何平均值在统计上低于冷冻前的NF50几何平均值
• 注意：效力下降与凝胶塌缩相关

图 4

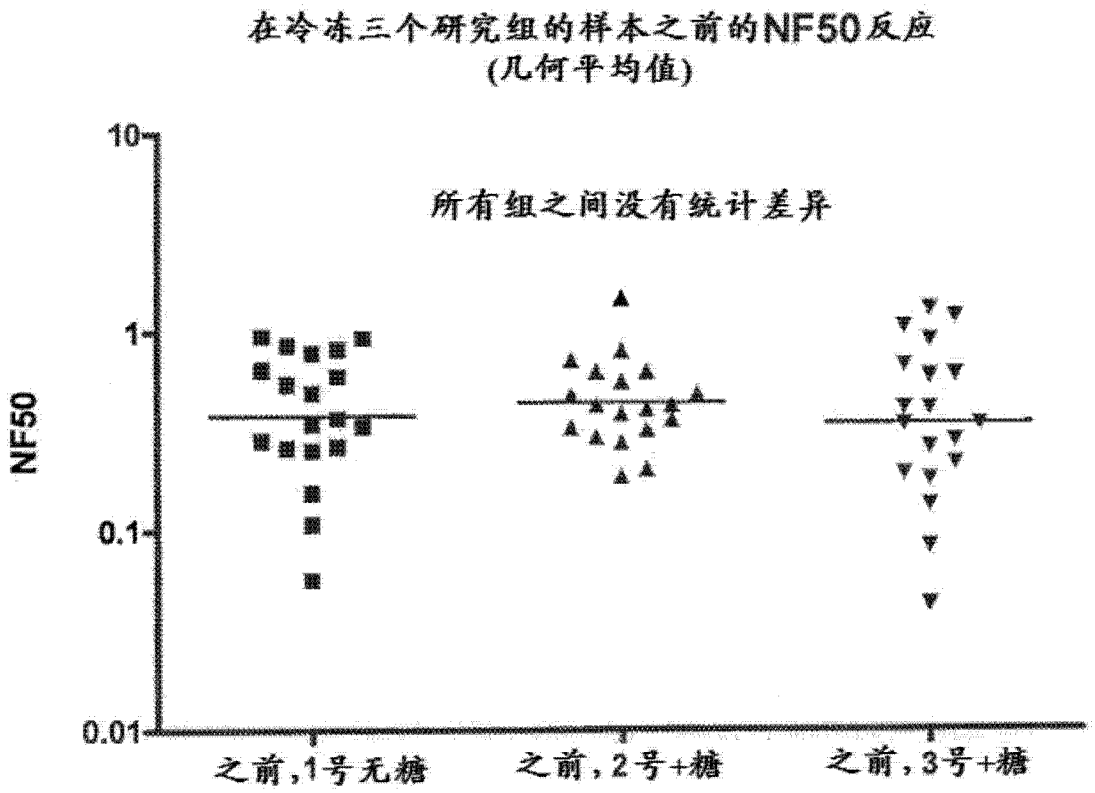

在冷冻三个研究组的样本之前的NF50反应
(几何平均值)
所有组之间没有统计差异
NF50
10
1
0.1
0.01
之前，1号无糖
之前，2号+糖
之前，3号+糖

图 5

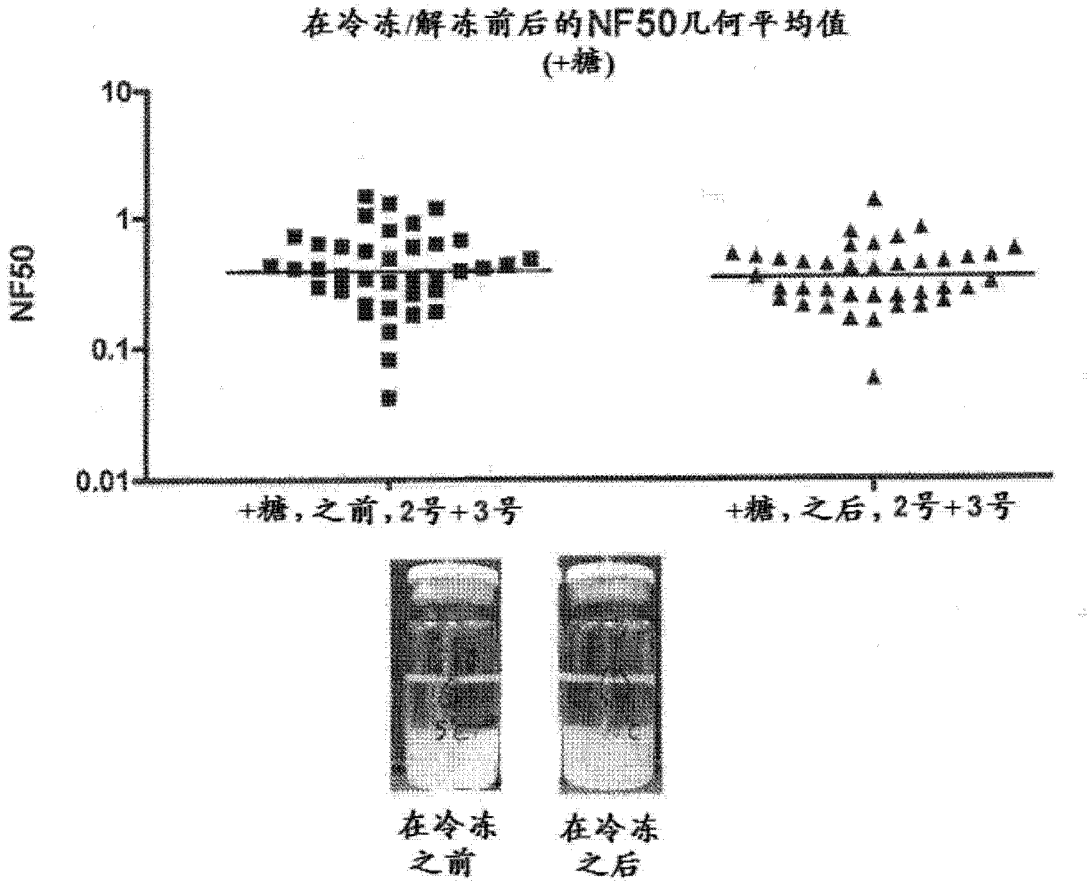

在冷冻/解冻前后的NF50几何平均值
(+糖)
NF50
10
1
0.1
0.01
+糖，之前，2号+3号
+糖，之后，2号+3号
在冷冻之前
在冷冻之后

图 6

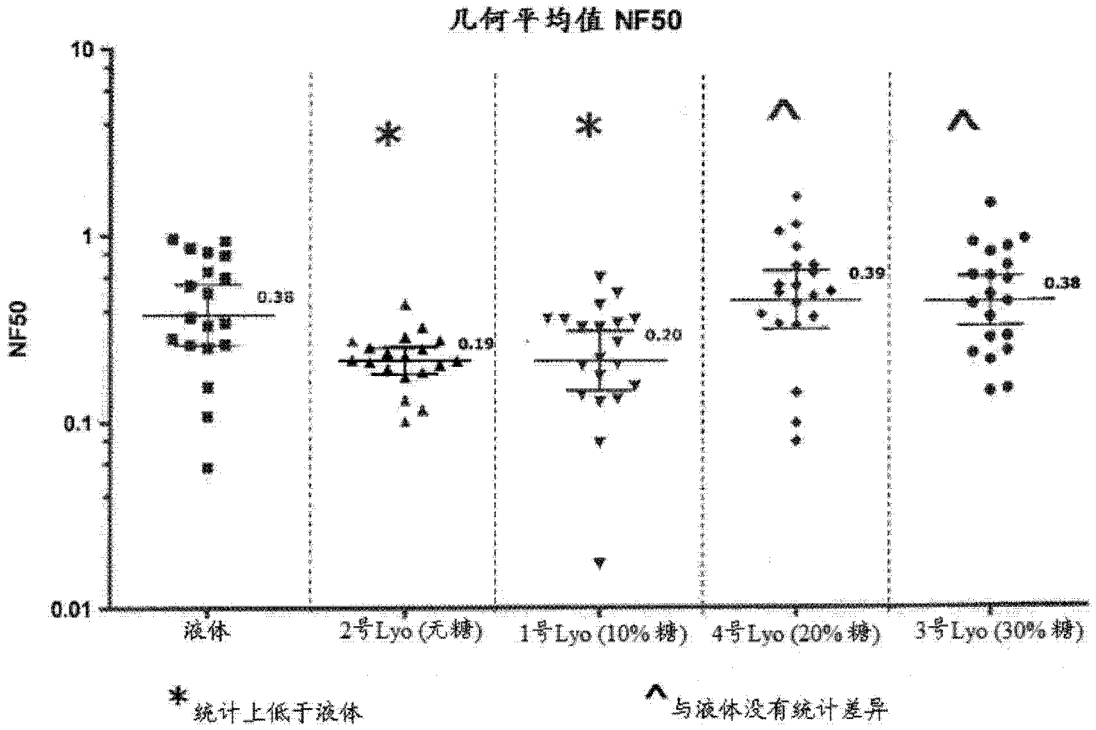

几何平均值 NF50
NF50
10
1
0.1
0.01
0.38
0.19
0.20
0.39
0.38
液体
2号Lyo (无糖)
1号Lyo (10% 糖)
4号Lyo (20% 糖)
3号Lyo (30% 糖)
*统计上低于液体
^与液体没有统计差异

图 7

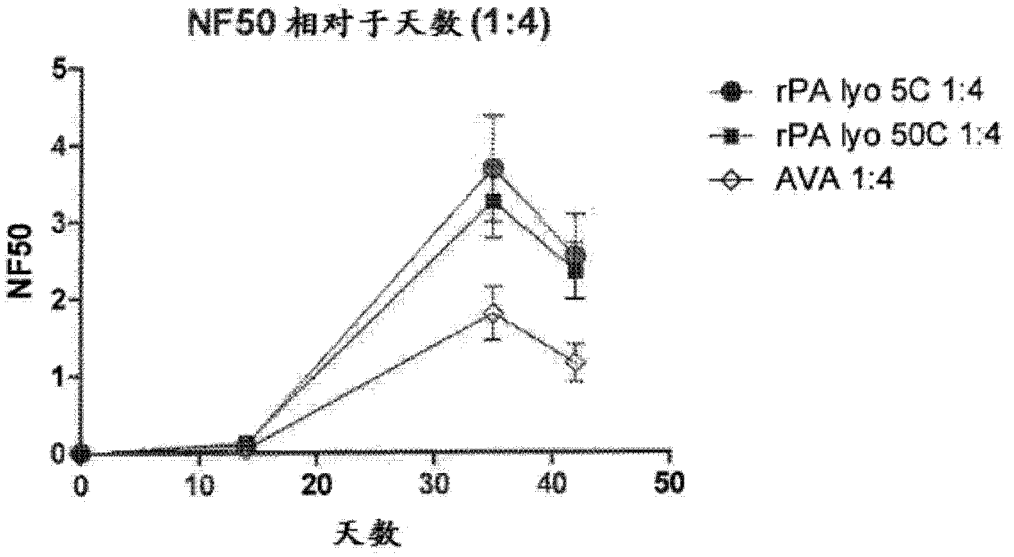

NF50 相对于天数 (1:4)
NF50
5
4
3
2
1
0
0
10
20
30
40
50
天数
rPA lyo 5C 1:4
rPA lyo 50C 1:4
AVA 1:4

图 8A

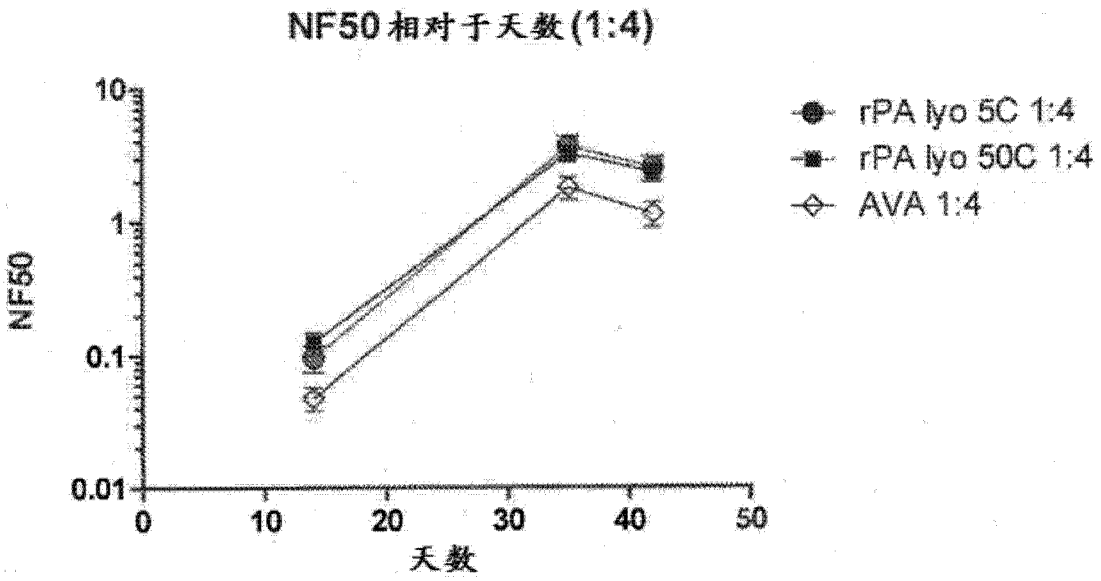

NF50 相对于天数 (1:4)
NF50
10
1
0.1
0.01
0
10
20
30
40
50
天数
rPA lyo 5C 1:4
rPA lyo 50C 1:4
AVA 1:4

图 8B

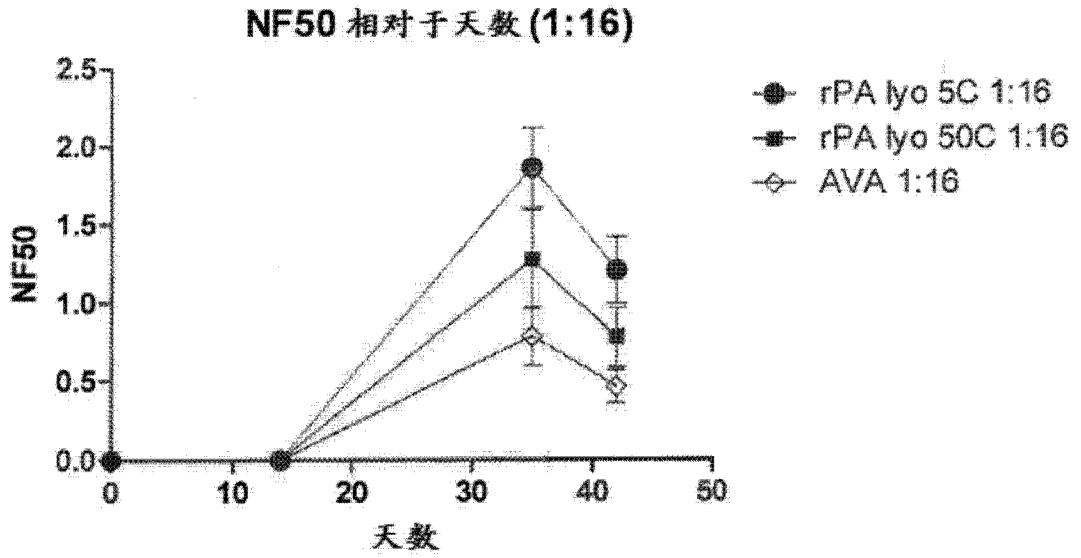

NF50 相对于天数 (1:16)
NF50
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
天数
rPA lyo 5C 1:16
rPA lyo 50C 1:16
AVA 1:16

图 9A

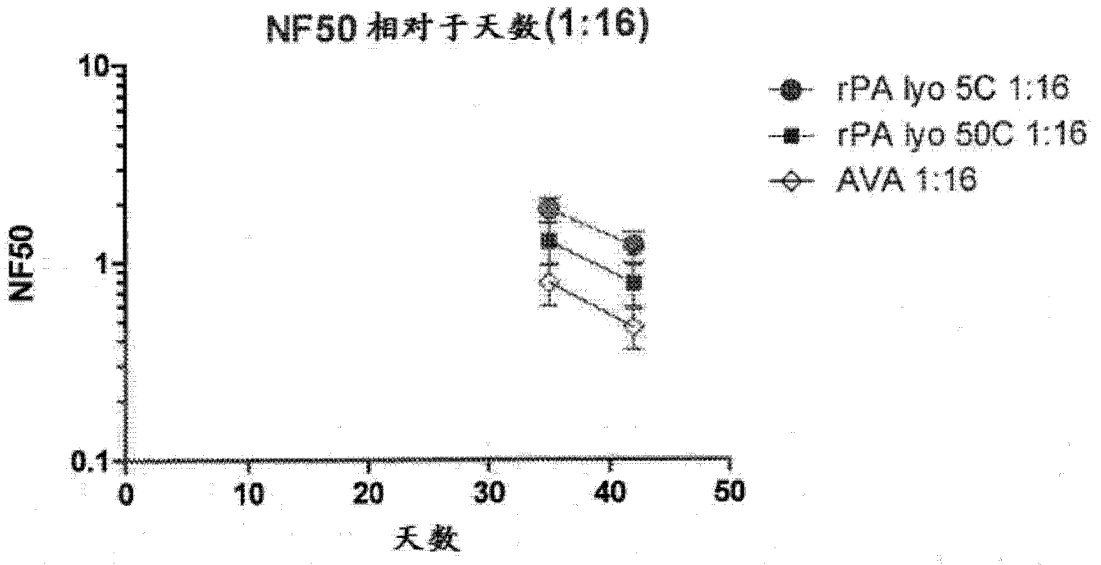

NF50 相对于天数(1:16)
NF50
10
1
0.1
0
10
20
30
40
50
天数
rPA lyo 5C 1:16
rPA lyo 50C 1:16
AVA 1:16

图 9B

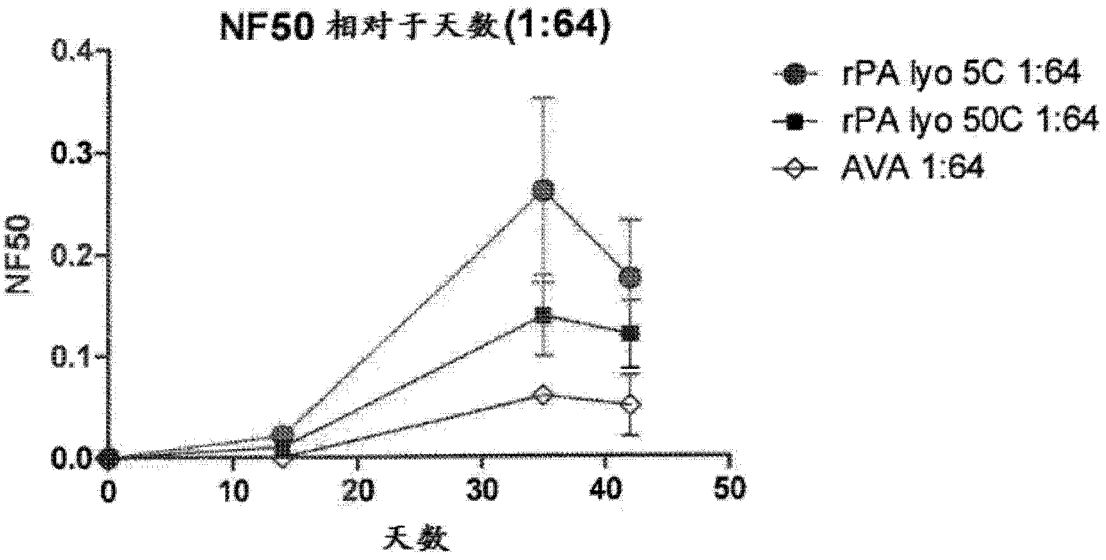

NF50 相对于天数(1:64)
NF50
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
天数
rPA lyo 5C 1:64
rPA lyo 50C 1:64
AVA 1:64

图 10A

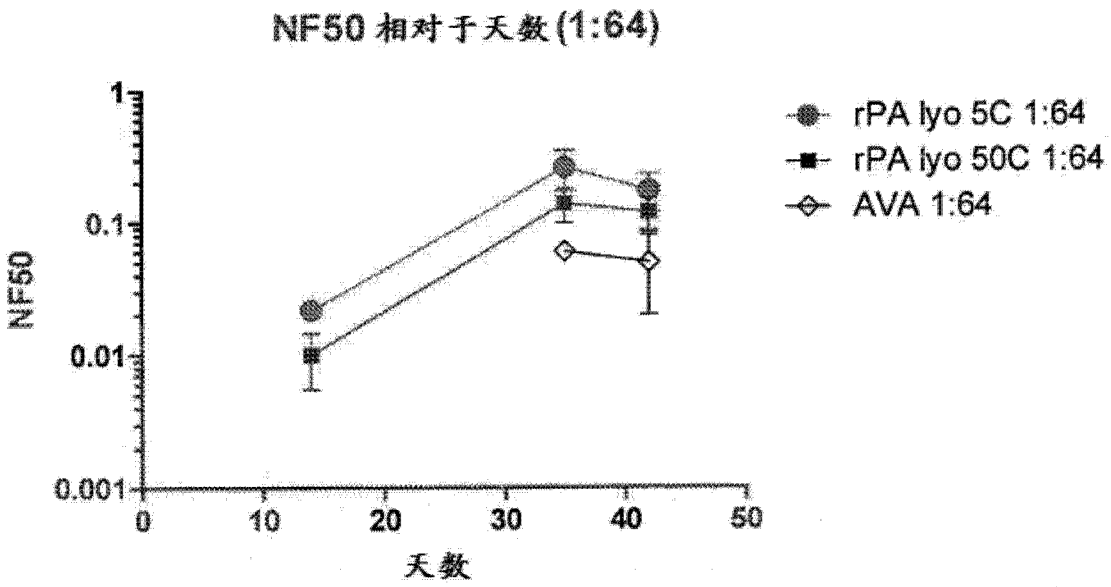

NF50 相对于天数(1:64)
rPA lyo 5C 1:64
rPA lyo 50C 1:64
AVA 1:64
NF50
1
0.1
0.01
0.001
0
10
20
30
40
50
天数

图 10B

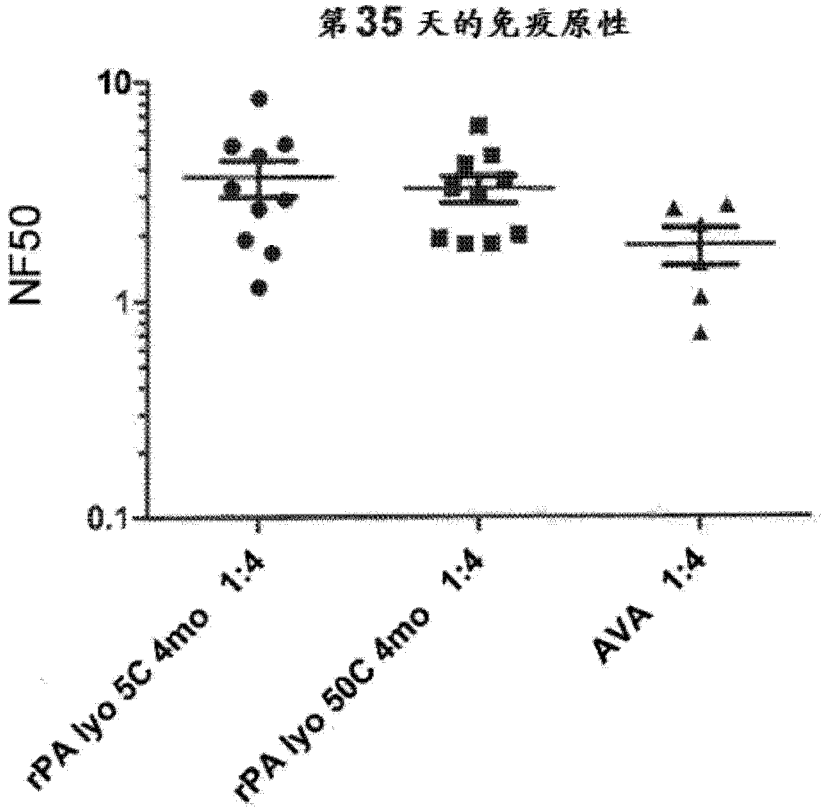

第35 天的免疫原性
NF50
10
1
0.1
rPA lyo 5C 4mo 1:4
rPA lyo 50C 4mo 1:4
AVA 1:4

图 11A

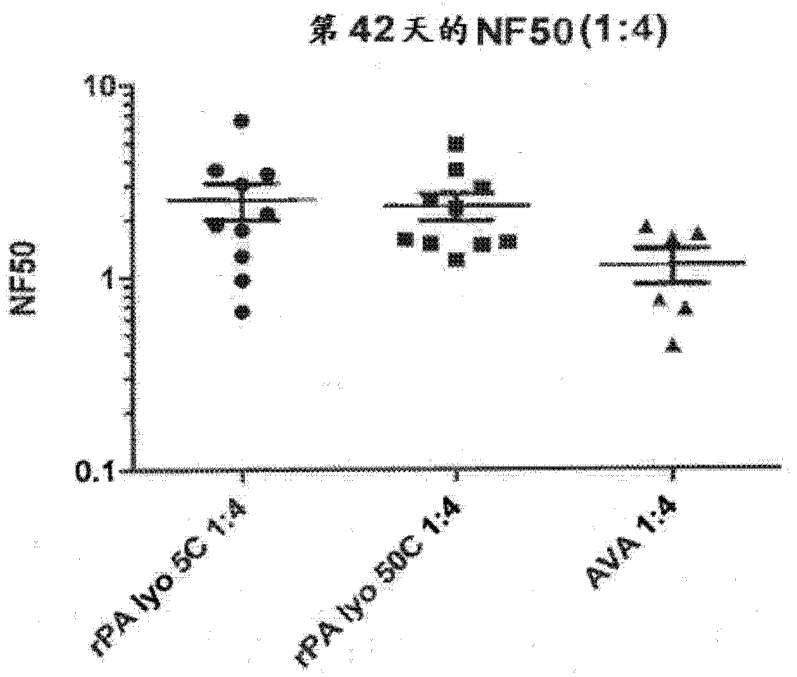

第42天的NF50(1:4)
NF50
10
1
0.1
rPA lyo 5C 1:4
rPA lyo 50C 1:4
AVA 1:4

图 11B

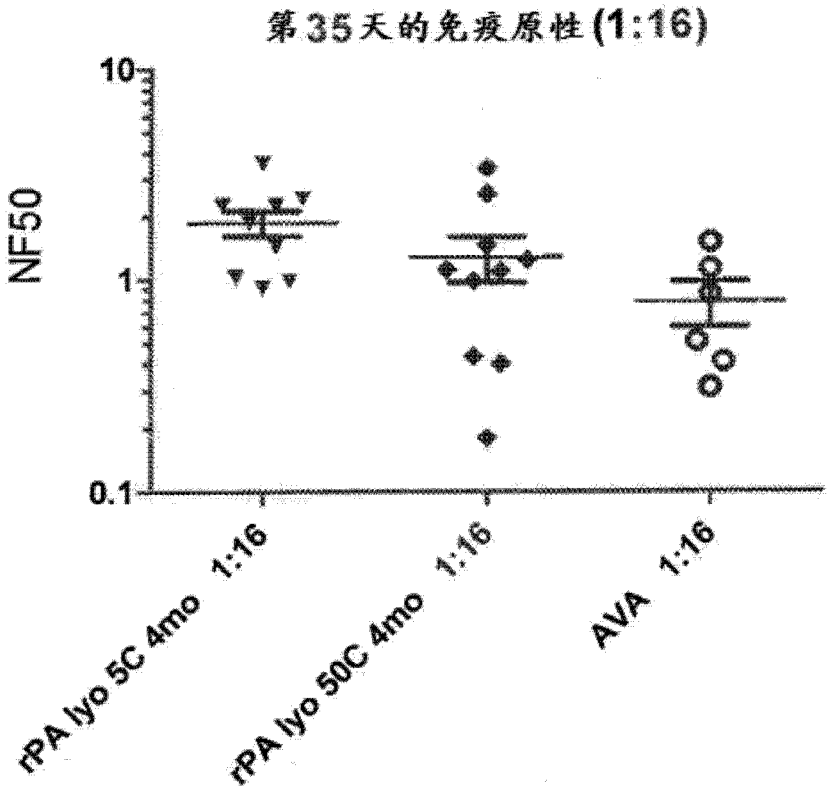

第35天的免疫原性(1:16)
NF50
10
1
0.1
rPA lyo 5C 4mo 1:16
rPA lyo 50C 4mo 1:16
AVA 1:16

图 12A

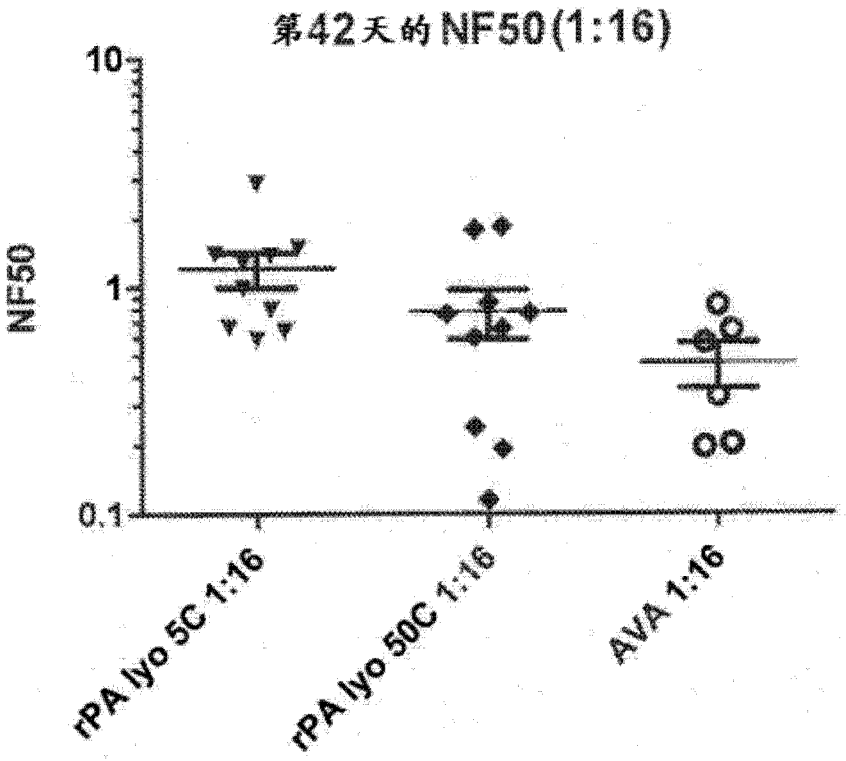

第42天的NF50(1:16)
NF50
10
1
0.1
rPA lyo 5C 1:16
rPA lyo 50C 1:16
AVA 1:16

图 12B

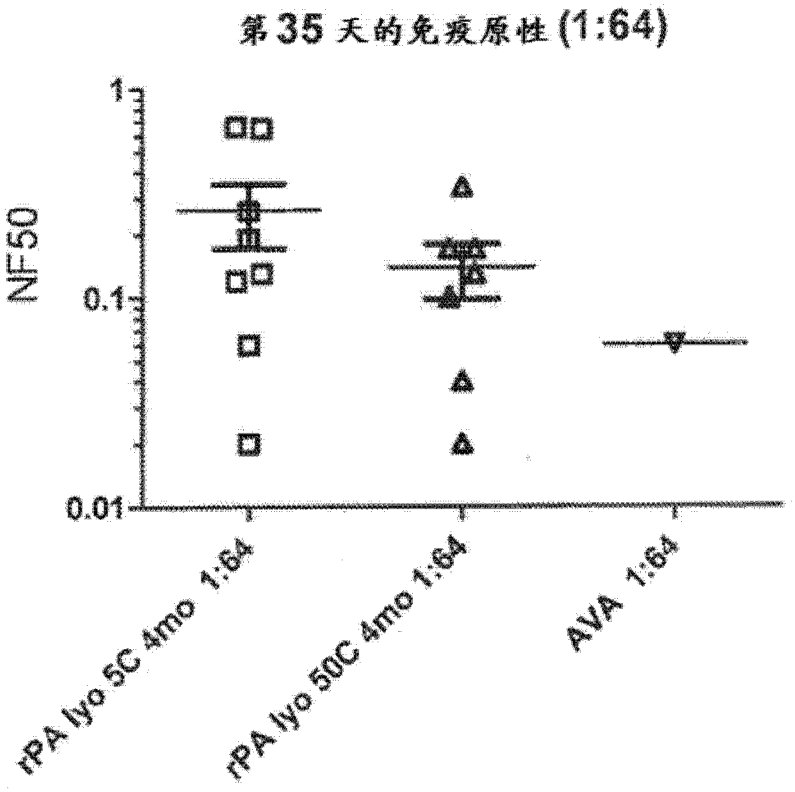

第35天的免疫原性(1:64)
NF50
1
0.1
0.01
rPA lyo 5C 4mo 1:64
rPA lyo 50C 4mo 1:64
AVA 1:64

图 13A

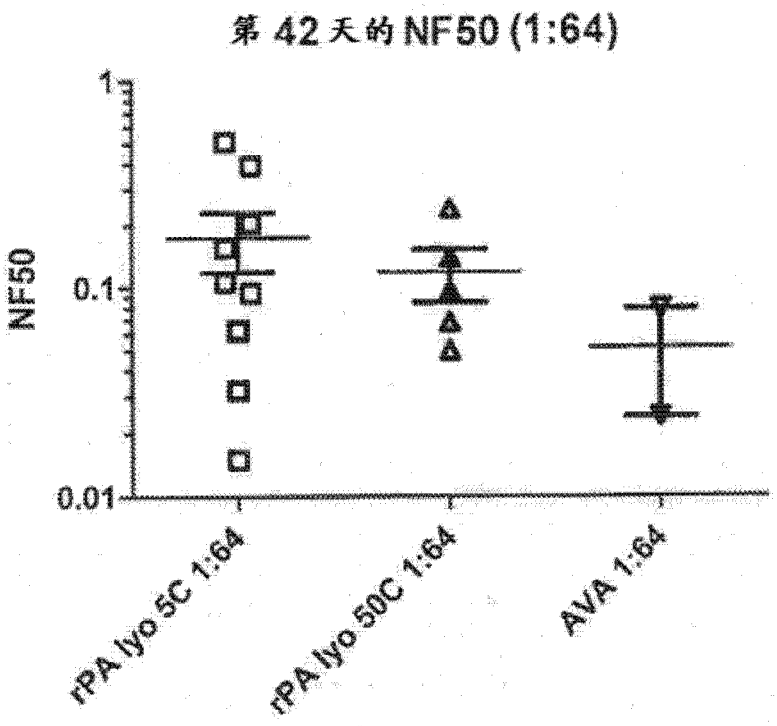

第 42 天的 NF50 (1:64)
NF50
1
0.1
0.01
rPA lyo 5C 1:64
rPA lyo 50C 1:64
AVA 1:64

图 13B

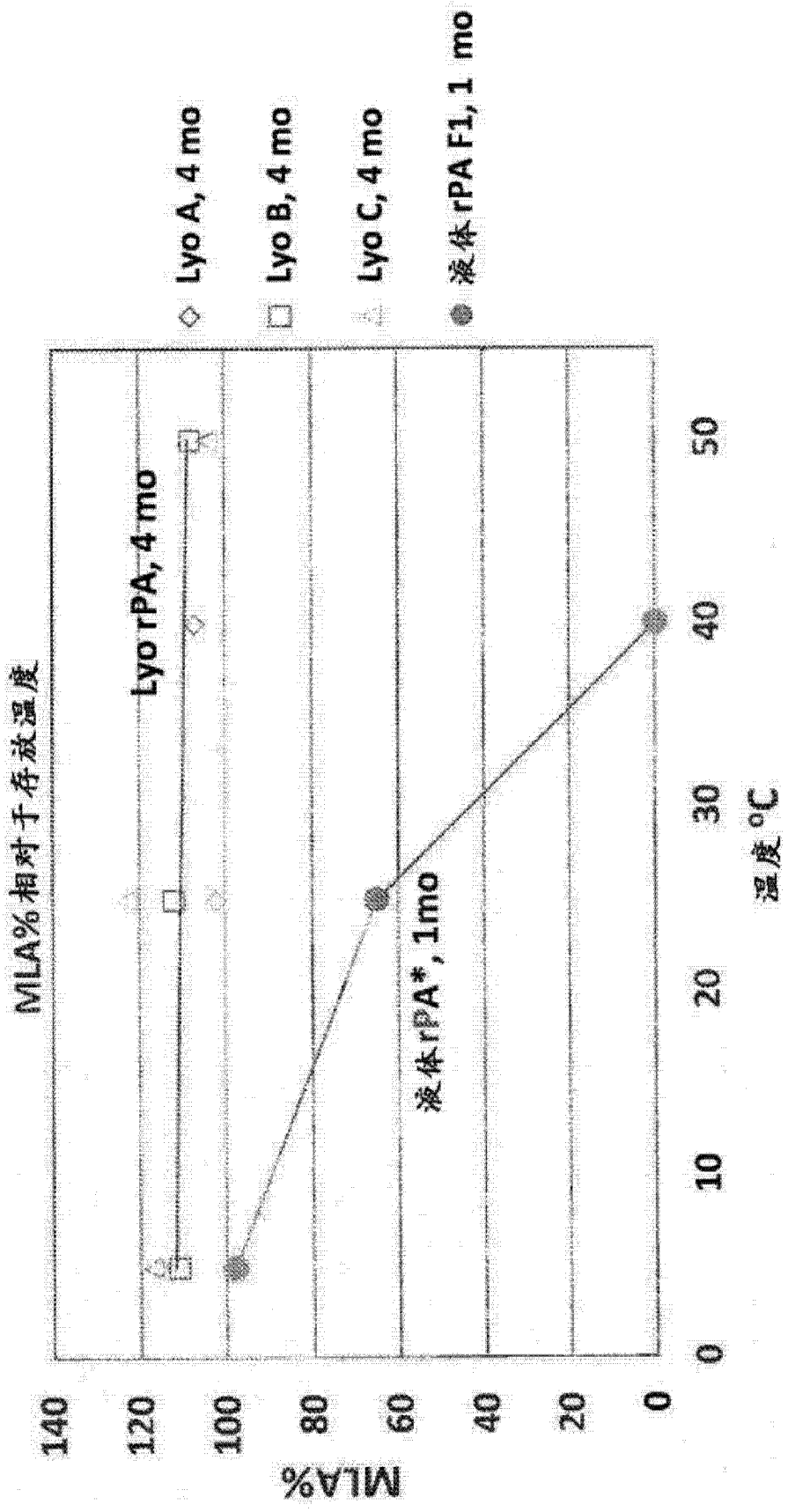

MLA%相对于存放温度
Lyo A, 4 mo
Lyo B, 4 mo
Lyo C, 4 mo
液体rPA F1, 1 mo
Lyo rPA, 4 mo
液体rPA*, 1mo
MLA%
温度°C
140
120
100
80
60
40
20
0
0
10
20
30
40
50

图 14

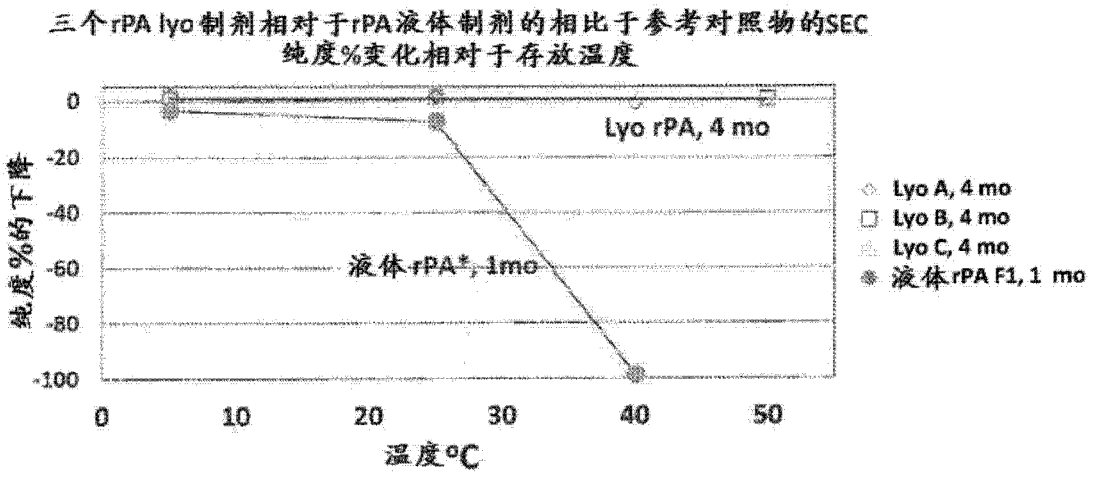

三个rPA lyo制剂相对于rPA液体制剂的相比于参考对照物的SEC
纯度%变化相对于存放温度
纯度%的下降
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
温度°C
Lyo rPA, 4 mo
液体rPA*, 1mo
Lyo A, 4 mo
Lyo B, 4 mo
Lyo C, 4 mo
液体rPA F1, 1 mo

图 15A

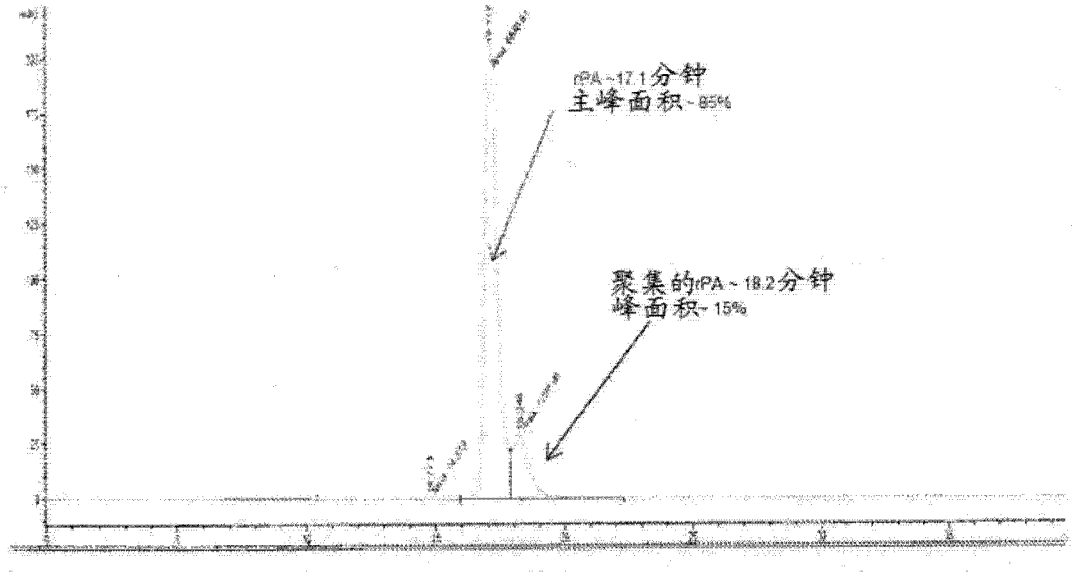

rPA~17.1分钟
主峰面积~85%
聚集的rPA~18.2分钟
峰面积~15%

图 15B

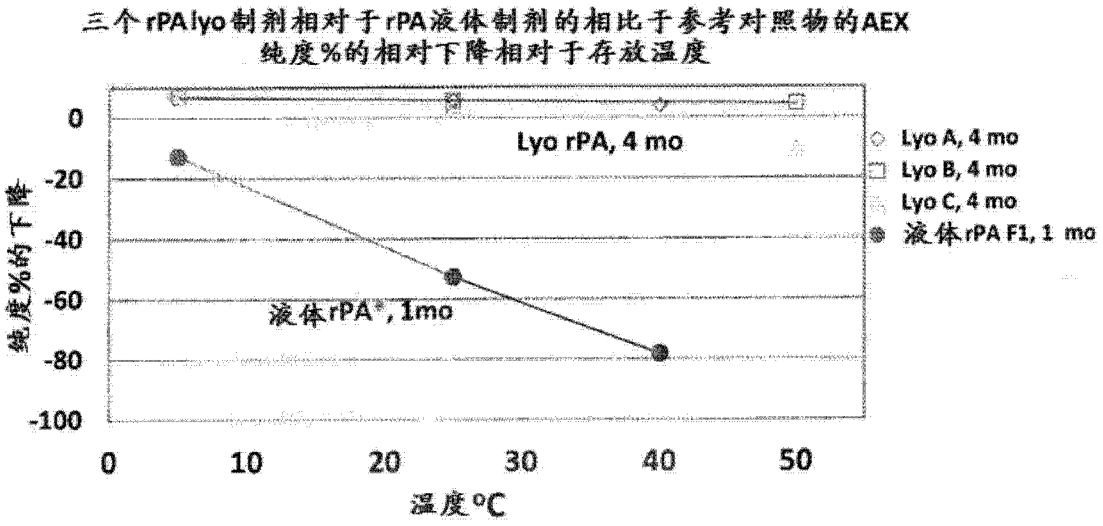

三个rPA lyo制剂相对于rPA液体制剂的相比于参考对照物的AEX
纯度%的相对下降相对于存放温度
纯度%的下降
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
温度°C
Lyo rPA, 4 mo
液体rPA*, 1mo
Lyo A, 4 mo
Lyo B, 4 mo
Lyo C, 4 mo
液体rPA F1, 1 mo

图 16A

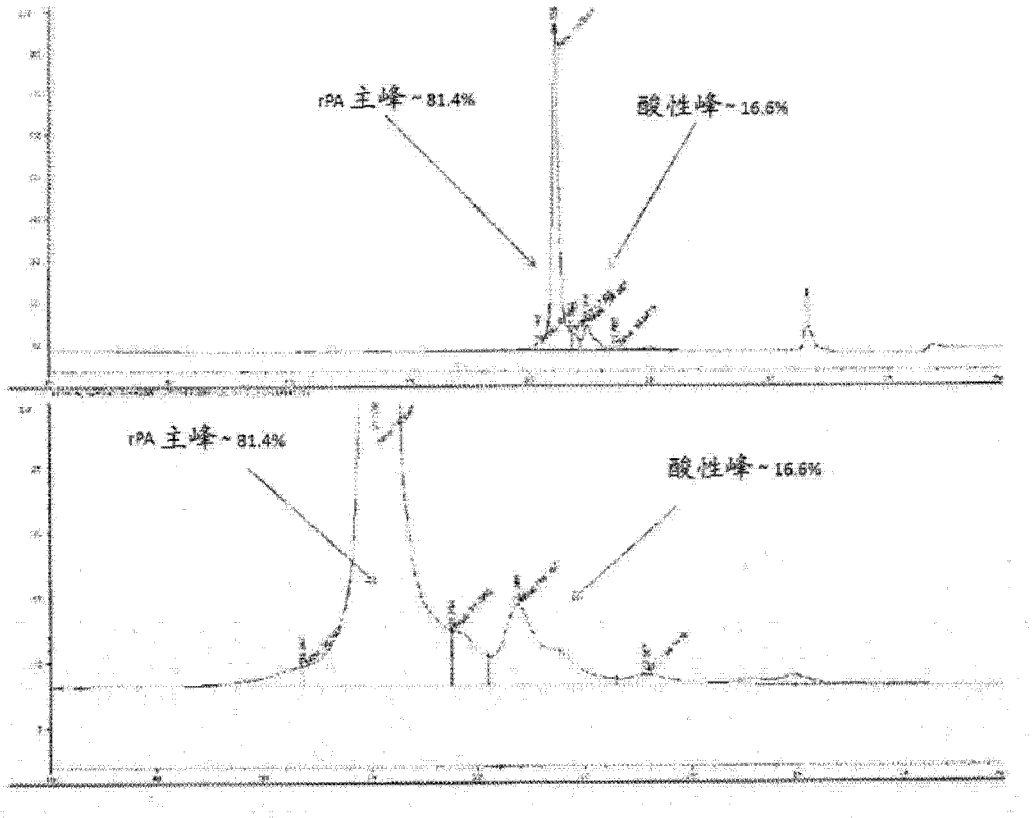

rPA 主峰~81.4%
酸性峰~16.6%
rPA 主峰~81.4%
酸性峰~16.6%

图 16B

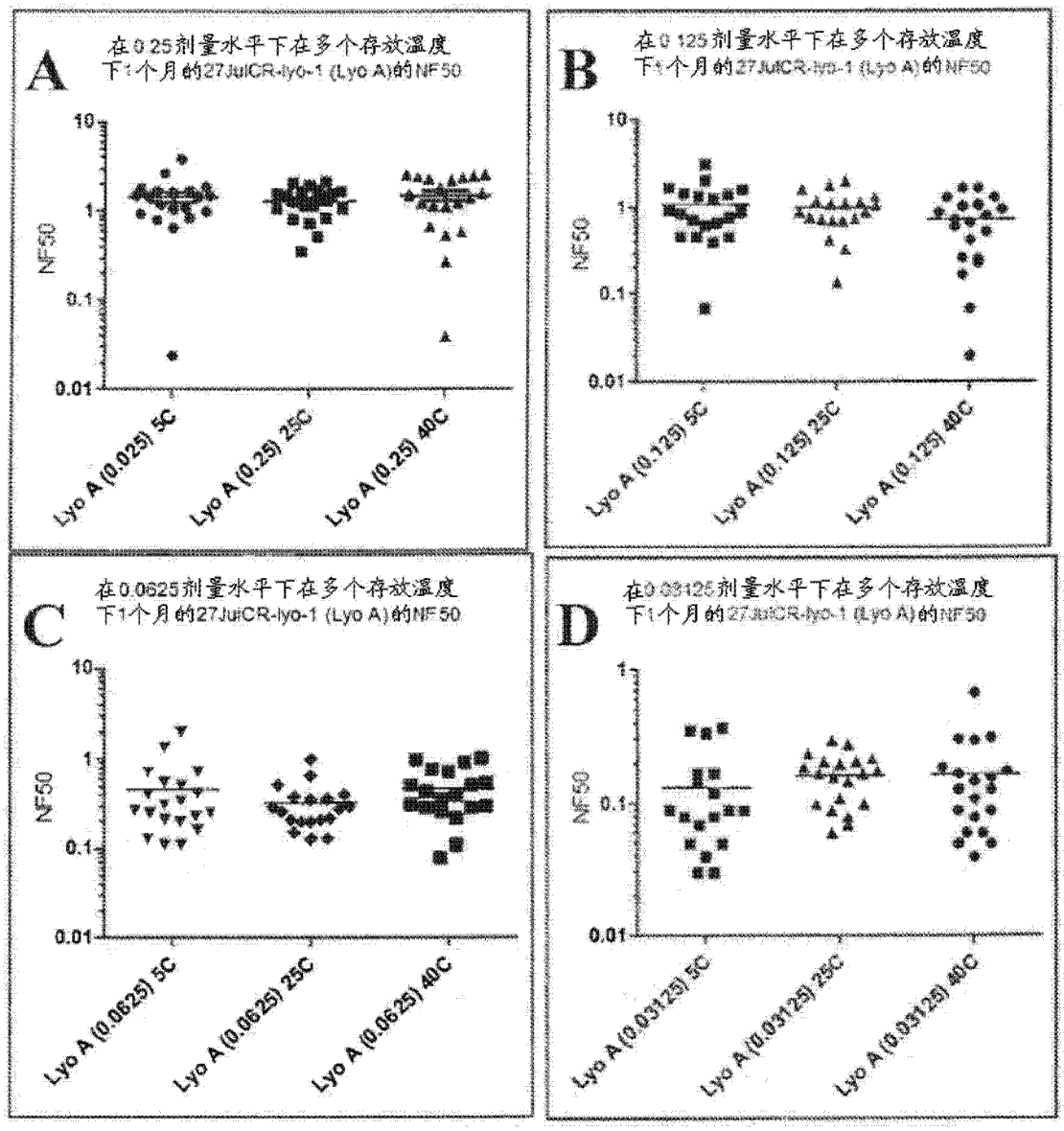

A
在0.25剂量水平下在多个存放温度
下1个月的27JulCR-lyo-1 (Lyo A)的NF50
NF50
10
1
0.1
0.01
Lyo A (0.025) 5C
Lyo A (0.25) 25C
Lyo A (0.25) 40C
B
在0.125剂量水平下在多个存放温度
下1个月的27JulCR-lyo-1 (Lyo A)的NF50
NF50
10
1
0.1
0.01
Lyo A (0.125) 5C
Lyo A (0.125) 25C
Lyo A (0.125) 40C
C
在0.0625剂量水平下在多个存放温度
下1个月的27JulCR-lyo-1 (Lyo A)的NF50
NF50
10
1
0.1
0.01
Lyo A (0.0625) 5C
Lyo A (0.0625) 25C
Lyo A (0.0625) 40C
D
在0.03125剂量水平下在多个存放温度
下1个月的27JulCR-lyo-1 (Lyo A)的NF50
NF50
1
0.1
0.01
Lyo A (0.03125) 5C
Lyo A (0.03125) 25C
Lyo A (0.03125) 40C

图 17A-D

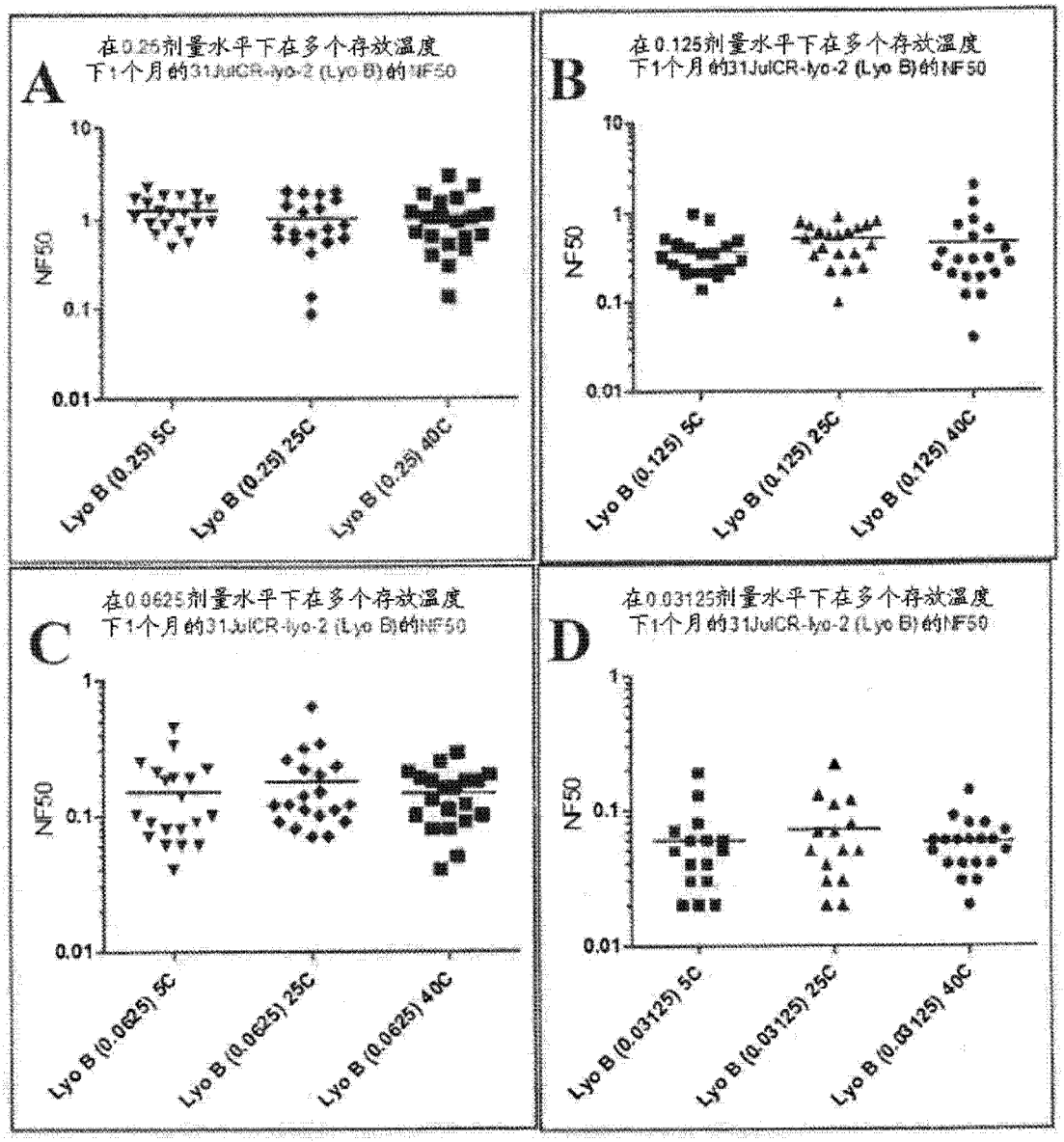

A
在0.25剂量水平下在多个存放温度
下1个月的31JulCR-lyo-2 (Lyo B)的NF50
NF50
10
1
0.1
0.01
Lyo B (0.25) 5C
Lyo B (0.25) 25C
Lyo B (0.25) 40C
B
在0.125剂量水平下在多个存放温度
下1个月的31JulCR-lyo-2 (Lyo B)的NF50
NF50
10
1
0.1
0.01
Lyo B (0.125) 5C
Lyo B (0.125) 25C
Lyo B (0.125) 40C
C
在0.0625剂量水平下在多个存放温度
下1个月的31JulCR-lyo-2 (Lyo B)的NF50
NF50
1
0.1
0.01
Lyo B (0.0625) 5C
Lyo B (0.0625) 25C
Lyo B (0.0625) 40C
D
在0.03125剂量水平下在多个存放温度
下1个月的31JulCR-lyo-2 (Lyo B)的NF50
NF50
1
0.1
0.01
Lyo B (0.03125) 5C
Lyo B (0.03125) 25C
Lyo B (0.03125) 40C

图 18A-D

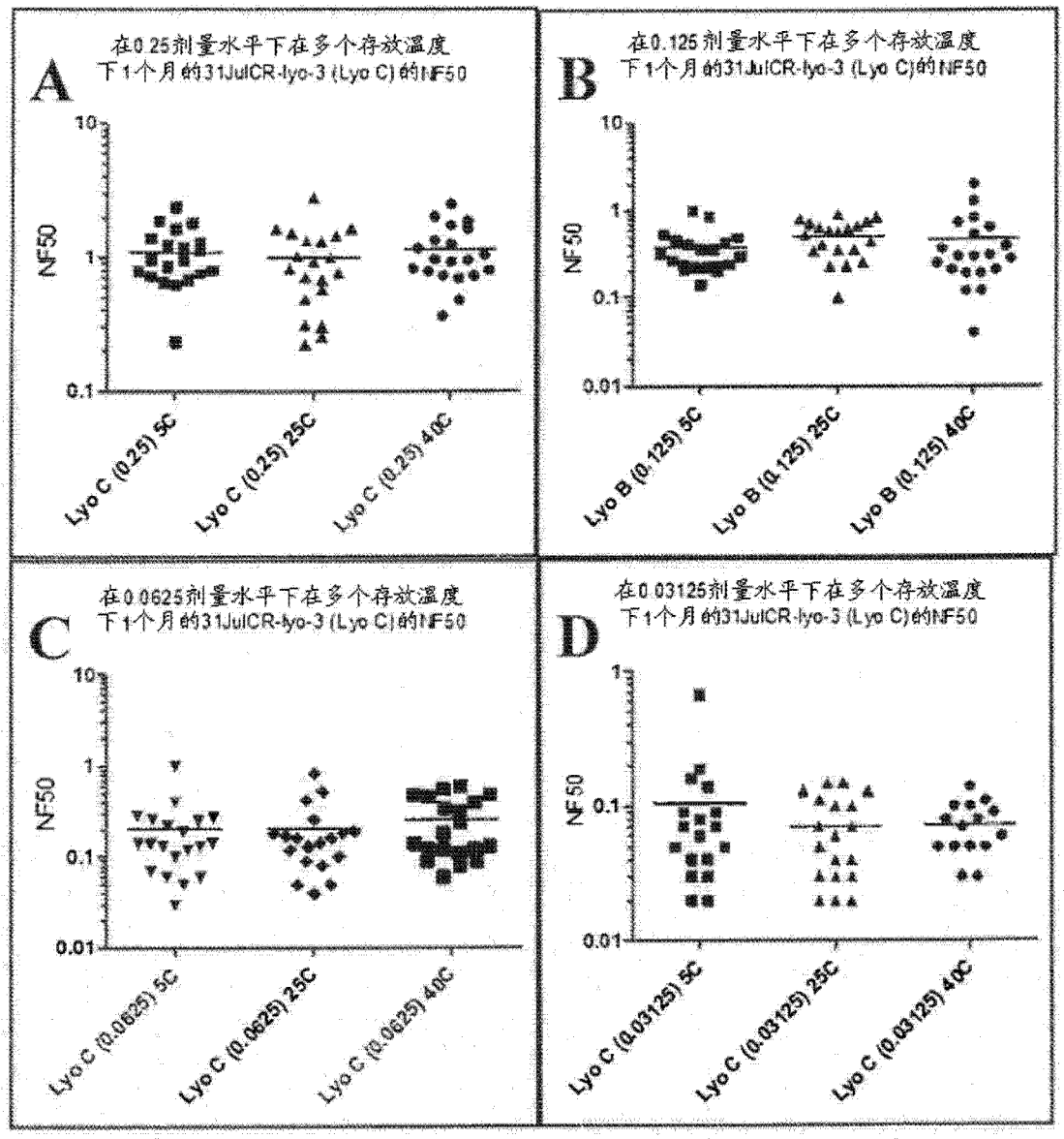

A
在0.25剂量水平下在多个存放温度
下1个月的31JulCR-lyo-3 (Lyo C)的NF50
NF50
10
1
0.1
Lyo C (0.25) 5C
Lyo C (0.25) 25C
Lyo C (0.25) 40C
B
在0.125剂量水平下在多个存放温度
下1个月的31JulCR-lyo-3 (Lyo C)的NF50
NF50
10
1
0.1
0.01
Lyo B (0.125) 5C
Lyo B (0.125) 25C
Lyo B (0.125) 40C
C
在0.0625剂量水平下在多个存放温度
下1个月的31JulCR-lyo-3 (Lyo C)的NF50
NF50
10
1
0.1
0.01
Lyo C (0.0625) 5C
Lyo C (0.0625) 25C
Lyo C (0.0625) 40C
D
在0.03125剂量水平下在多个存放温度
下1个月的31JulCR-lyo-3 (Lyo C)的NF50
NF50
1
0.1
0.01
Lyo C (0.03125) 5C
Lyo C (0.03125) 25C
Lyo C (0.03125) 40C

图 19A-D

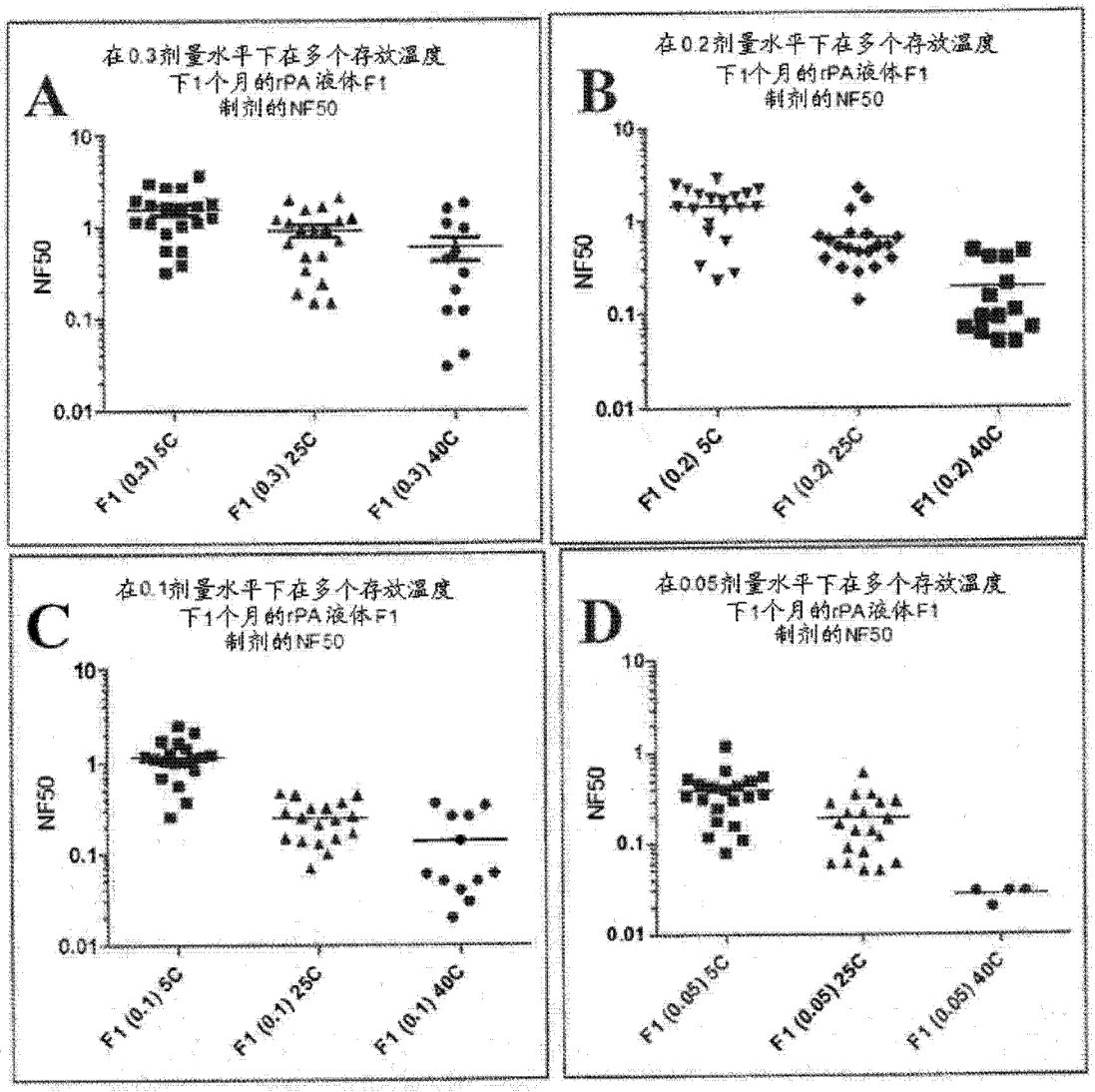

A
在0.3剂量水平下在多个存放温度
下1个月的rPA液体F1
制剂的NF50
NF50
10
1
0.1
0.01
F1 (0.3) 5C
F1 (0.3) 25C
F1 (0.3) 40C
B
在0.2剂量水平下在多个存放温度
下1个月的rPA液体F1
制剂的NF50
NF50
10
1
0.1
0.01
F1 (0.2) 5C
F1 (0.2) 25C
F1 (0.2) 40C
C
在0.1剂量水平下在多个存放温度
下1个月的rPA液体F1
制剂的NF50
NF50
10
1
0.1
0.01
F1 (0.1) 5C
F1 (0.1) 25C
F1 (0.1) 40C
D
在0.05剂量水平下在多个存放温度
下1个月的rPA液体F1
制剂的NF50
NF50
10
1
0.1
0.01
F1 (0.05) 5C
F1 (0.05) 25C
F1 (0.05) 40C

图 20A-D

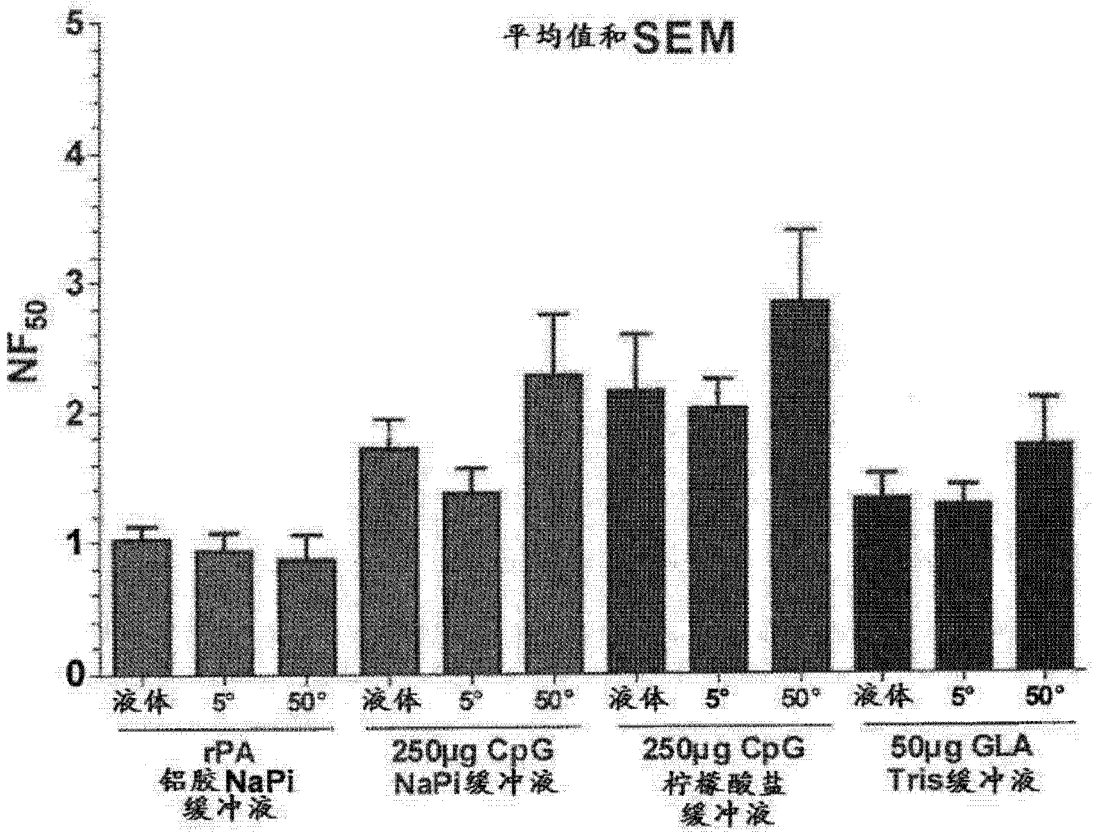

平均值和SEM
NF50
0
1
2
3
4
5
液体
5°
50°
液体
5°
50°
液体
5°
50°
液体
5°
50°
rPA
铝胺NaPi
缓冲液
250μg CpG
NaPi缓冲液
250μg CpG
柠檬酸盐
缓冲液
50μg GLA
Tris缓冲液

图 21

Abstract

Formulations of vaccine antigen, such as anthrax protective antigen, are provided that are stable after undergoing freeze and thaw conditions. Methods of using the formulations to prepare vaccine are also provided. Vaccines comprising the formulations are useful, for example, to protect against, inhibit or alleviate a disease or infection, such as related to anthrax infection.