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(54) Title: IMMUNOREACTIVE FRANCISELLA TULARENSIS ANTIGENS

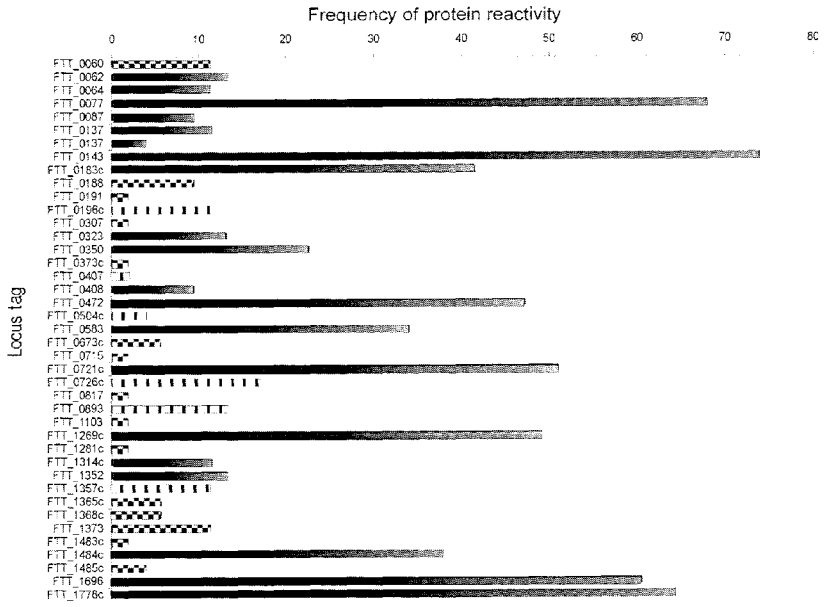


FIG. 8

(57) Abstract: The present invention provides sets of biomarkers for tularaemia comprising one or more proteins selected from the group consisting of: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 50S ribosomal protein L7/L12, 30S ribosomal protein S1, DNA-directed RNA polymerase alpha subunit, Acetyl-CoA carboxylase, Outer membrane associated protein FopA, Peroxidase/catalase, Chaperone protein DnaK, Pyruvate dehydrogenase E2 component, Chaperone protein groEL, Hypothetical membrane protein, and any combination thereof, and their use as correlates of protection and for evaluating vaccine efficacy.

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IMMUNOREACTIVE FRANCISELLA TULARENSIS ANTIGENS

Cross-reference to Related Applications

This application claims the benefit of United States Provisional Patent Application USSN 61/433,385 filed January 17, 2011, the entire contents of which are herein incorporated by reference.

Field of the Invention

The present invention relates to immunoreactive *Francisella tularensis* antigens and uses thereof. More specifically, the invention relates to immunoreactive *Francisella tularensis* antigens and their uses as correlates of protection.

BACKGROUND OF THE INVENTION

Tularemia is a disease caused by the Gram-negative facultative intracellular bacterium, *Francisella tularensis. F. tularensis* is pathogenic for many mammalian species including humans, causing a spectrum of diseases collectively called tularemia. Tularemia has been reported as a clinical infection in primates in many temperate climates across the world. Several subspecies exist, with the most clinically relevant subspecies being *holarctica* and *tularensis,* commonly denoted Type B and A strains, respectively (Sjostedt, 2001). The subspecies *tularensis* (Type A) is endemic only to North America. Mortality rates of up to 60% have been reported for untreated human cases of disseminated infection caused by Type A strains of the pathogen (Dienst, 1963). The subspecies *holarctica* (Type B), endemic to both Europe and North America, is associated with a less severe clinical manifestation and lower mortality rates. Type B strains are responsible for almost all European cases of tularemia (Sjostedt, 2007).

In the 1950's a live vaccine strain (LVS) was empirically derived from a Soviet strain, S15, and was found to protect humans to some degree against subsequent exposure to Type A strains of the pathogen (Hornick & Eigelsbach, 1966). For example, when LVS replaced killed bacteria as the vaccine at USAMRIID, the incidence of respiratory infections among at risk personnel was significantly reduced (Burke, 1977; Eigelsbach et al, 1967). Human volunteer LVS vaccination studies were conducted under the Operation Whitecoat (OW) program in the 1950s. These data showed that LVS administered by scarification was 25-100% effective against aerosol challenge with SCHU S4 (Hornick and Eigelsbach, 1966). In addition, all

vaccinees were shown to seroconvert to an undefined set of *Francisella* antigens, but no immunologic correlation was established with the protective status of the host.

Due to renewed concerns regarding its potential use in bioterrorism, there has been an increased interest during the past decade in licensing a tularemia vaccine for general use. LVS remains the only tularemia vaccine to have shown efficacy in humans. LVS has been successfully used in Europe and the USA to protect tularemia researchers against Type A strains (Oyston et al, 2005; Conlan, 2004; Titball & Oyston, 2003). The LVS NDBR101 Lot 11 has also been administered to Swedish laboratory staff, and during four decades of active tularemia research, there have been very few reported cases of laboratory-acquired infections in vaccinated individuals. However, the absence of a correlate of protection is one of several significant barriers to the licensure of LVS.

During OW and subsequent studies, no correlation between the antibody titre to protein antigens in humans and level of protection against challenge with virulent *F. tularensis* was observed (Hornick & Eigelsbach, 1966; Saslaw & Carhart, 1961; Saslaw & Eigelsbach, 1961a; Saslaw & Eigelsbach, 1961b). In the past decade, a handful of studies have surveyed the repertoire of murine antibodies generated in response to LVS vaccination (Havlasova et al, 2005; Sundaresh et al, 2007; Eyles et al, 2007) and human tularemia infection (Havlasova et al, 2002; Janovska et al, 2007a; Janovska et al, 2007b). Some of these studies measured only antibody titres in the subjects, while others used immunoproteomics on sera from LVS infected mice; however, the applicability of findings in animal models, especially mice, in humans is questionable.

Ethical considerations prevent tularaemia vaccine efficacy studies in humans; thus, evaluation of vaccines must be conducted in animal models of tularaemia using the FDA Animal Rule. The Animal Rule allows demonstration of the efficacy of vaccines that cannot be tested in human clinical trials via efficacy studies in animals. Application of the Animal Rule to tularaemia vaccine candidates would be facilitated by immunological correlate of protection or vaccine marker to bridge efficacy in animals to immunogenicity in humans.

Thus, there remains a need in the art for a correlate of protection to establish a positive relationship between efficacy of tularaemia vaccine in animals and humans.

SUMMARY OF THE INVENTION

The present invention relates to immunoreactive *Francisella tularensis* antigens and uses thereof. More specifically, the invention relates to immunoreactive *Francisella tularensis* antigens and their uses as correlates of protection.

Thus, the present invention provides a set of biomarkers for tularaemia, selected from the group consisting of: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 50S ribosomal protein L7/L12, 30S ribosomal protein S1, DNA-directed RNA polymerase alpha subunit, Acetyl-CoA carboxylase, Outer membrane associated protein FopA, Peroxidase/catalase, Chaperone protein DnaK, Pyruvate dehydrogenase E2 component, Chaperone protein groEL, Hypothetical membrane protein, and any combination thereof. The biomarkers of the present invention may be reactive with tularaemia-exposed sera at a frequency of about 30-100%.

The present invention also provides a method of evaluating immunity against tularaemia in a subject, comprising:

a. contacting serum from the subject with the set of biomarkers of the present invention;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. determining the protection against tularaemia based on immunoreactivity to the biomarkers.

The present invention further provides a method of evaluating vaccine efficacy, comprising:

a. contacting serum from a vaccinated model of tularaemia with the biomarkers of the present invention;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. correlating the immunoreactivity to immunoprotective status of the model; and

d. predicting vaccine efficacy in human based on the correlation of step c.

Recent studies in the murine model of tularemia show that adaptive host defense against *F. tularensis* is likely mediated by both cell-mediated immunity (CMI) and humoral immunity (Tarnvik, 1989; Elkins et al, 2003; Kirimanjeswara et al, 2008). Although CMI is thought to be the most essential mechanism in host defense against Type A *Francisella,* specific antibody

responses are mounted during natural *Francisella* infections or following vaccination (Dennis et al, 2001; Saslaw & Carhart, 1961; Carlsson et al, 1979; Viljanen et al, 1983). Patients that recover from types A and B *Francisella* infections are rarely reported to show signs of disease following a second exposure, and therefore could be considered a group that is protected from further challenge. Therefore, this allows the comparison of the repertoire of antibodies between infected, but presumably protected individuals, and vaccinated volunteers whose protective status is unknown.

The present invention makes use of immunoproteomics to identify the antigenic proteins from human tularemia patients and LVS vaccinees, including sera from subjects in FDA clinical trial of LVS. A gel-based immunoproteomics approach was used to identify immunoreactive proteins generated in response to LVS vaccination of mice, rabbits, non-human primates (NHP), and humans. Proteins observed to be immunoreactive with the majority of sera within a study group or across species were identified by tandem mass spectrometry. The results showed that tularaemia infection or LVS vaccination stimulates the generation of antibodies towards a small subset of the *Francisella* proteome. Specifically, eleven proteins were downselected as commonly reactive antigens, reactive with both patient and vaccinee sera, with a minimum frequency of 30 % (i.e., proteins were observed to be reactive with minimum 30 % of sera screened). These include dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FT_0077), 50S ribosomal protein L7/L12 (FTT_0143), 30S ribosomal protein S1 (FTT_0183), DNA-directed RNA polymerase alpha subunit (FTT_0350), Acetyl-CoA carboxylase (FTT0472), Outer membrane associated protein FopA (FTT_0583), Peroxidase/catalase FTT_0721c), Chaperone protein DnaK (FTT_1269c), Pyruvate dehydrogenase E2 component (FTT_1484c), Chaperone protein groEL (FTT1696), Hypothetical membrane protein (FTT_1778c). Similarities were observed in the repertoire of immunoreactive proteins generated by LVS vaccination across species. No single immunoreactive proteins correlated with protection of the host; however, a combination of the immunoreactive proteins or a qualitative immune response to several immunogenic proteins may provide a correlate of protection.

Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only, as various changes and modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

FIGURE 1 shows Western blots probed with sera from Type B patients. 100 µg of SCHU S4 Δ*wbtI* (O-antigen negative) was used as the antigen, with first dimension separation in the pH range 4-7. Blots were probed with 1:500 dilution of human sera.

FIGURE 2 shows Western blots probed with sera from Type A patients. Patient identification numbers are indicated, above each blot. 100 µg of SCHU S4 Δ*wbtI* (O-antigen negative) was used as the antigen, with first dimension separation in the pH range 4-7. Blots were probed with 1:500 dilution of human sera.

FIGURE 3 shows Western blots probed with sera from LVS vaccinees (Umea). Patient identification numbers are indicated above each blot image. D0, indicates sera drawn on the day of vaccination, d42 denotes sera drawn 42 days post vaccination. 100 µg of SCHU S4 Δ*wbtI* (O-antigen negative) was used as the antigen, with first dimension separation in the pH range 4-7. Blots were probed with 1:500 dilution of human sera.

FIGURE 4 shows Western blots probed with sera from LVS vaccinaees (Baylor). 100 µg of SCHU S4 Δ*wbtI* (O-antigen negative) was used as the antigen, with first dimension separation in the pH range 4-7. Blots were probed with 1:500 dilution of human sera.

FIGURE 5 shows representative Two-dimensional Western blots probed with sera from tularemia patients and LVS vaccinees. 100 µg of SCHU S4 Δ*wbtI* (O-antigen negative) was used as the antigen, with first dimension separation in the pH range 4-7. Blots were probed with 1:500 dilution of human sera as follows (a) Control 1, (b) Type B tularemia patient serum number 1671, (c)) Type A tularemia patient serum number MV758, (d) Type A tularemia patient serum number MV756, (e) NDBR lot 11 LVS vaccinee control serum number 110d0, (f) NDBR lot 11 LVS vaccinee day 42 post vaccination serum number 201d42, (g) DVC lot 17 LVS vaccine pre-vaccination serum number 200139603(paired id 02832), (h)) DVC lot 17 LVS vaccine day 42 post-vaccination serum number 2001362794 (paired id 02832). The complete set of Western blot images are shown in supplementary data Figures 1-4.

FIGURE 6 shows a two-dimensional gel resolving the SCHU S4 proteome. Protein stained 2D-gel, separating SCHU S4 protein lysates in the pH range 4-7, used for alignment of 2D Western blots and identification of immunoreactive proteins. Identified proteins are annotated with their locus tags, and listed in full in Table 2.

FIGURE 7 is a summary of immunoreactive proteins. FIGURE 7A shows the immunoreactive proteins and their relative intensity values observed for each serum screened. The bar chart below this matrix shows the total intensity of the immunoreactive proteins for each serum sample screened. FIGURE 7B shows the predicted subcellular locations for the identified immunoreactive proteins. These were determined using the PSORT1b algorithm, as described in the methods. FIGURE 7C shows the Clusters of Orthologous Group classification of the identified immunoreactive proteins.

FIGURE 8 is a bar chart representation of the frequency with which each immunoreactive protein was observed in the sera screened. Square fill indicates immunoreactive proteins that were only observed to react with sera from tularemia patients. Vertical line fill indicates proteins that were observed to be immunoreactive only with sera from LVS vaccinees. Black fill indicates immunoreactive proteins that were observed to be reactive with sera from both tularemia patients and vaccinees.

FIGURE 9 shows representative 2D Western blots probed with sera from LVS vaccinated non-human primates (Rhesus macaque LVS efficacy vaccination study). Shown are representative 2D-Western blots probed with sera from animal 0607036, seven days pre- LVS vaccination (FIGURE 9A); animal 0607036, 28 days post-LVS vaccination (scarification) (FIGURE 9B); animal 0602084, 28 days post LVS vaccination (subcutaneous) (FIGURE 9C); animal RQ7796 56 days post LVS vaccination (scarification) (FIGURE 9D); and animal RQ7459, 56 days post LVS vaccination (subcutaneous) (FIGURE 9E). The complete set of blots, including pre and post vaccination for each animal, are shown in FIGURE 10.

FIGURE 10 shows 2D-Western blots of sera from LVS-vaccinated non human primate. Group 3 (FIGURE 10A) animals were vaccinated via scarification and challenged 35 days post-vaccination. Group 4 (FIGURE 10B) animals were vaccinated subcutaneously and challenged 35 days post-vaccination. Group 5 (FIGURE 10C) animals were vaccinated via scarification and challenged 63 days post-vaccination. Group 6 (FIGURE 10D) animals were vaccinated subcutaneously and challenged 63 days post-vaccination. Sera are paired, with the left hand blot of each pair probed with pre-vaccinated sera and the right hand blot of each pair probed with sera drawn post-vaccination.

FIGURE 11 is a matrix of immunoreactive proteins for non-human primate LVS efficacy study. The blot images were analyzed using PDQuest software and the comparative intensity values for each immunoreactive area measured. Shading indicates the degree of observed immunoreactivity, from no observed immunoreactivity (□), to intense immunoreactivity (■).The animal numbers shaded in grey indicate LVS vaccinated survivors of SCHU S4 challenge.

FIGURE 12 is a bar chart showing total intensity of observed immunoreactivity for LVS vaccinated non-human primates. Animals are grouped by route of vaccination, and by day sera was collected post vaccination. Asterisk (*) indicates animals that survived challenge.

FIGURE 13 shows 2D Western blots probed with sera from LVS-vaccinated rabbits. New Zealand White rabbits Animal number 7, sera drawn seven days pre-vaccination (FIGURE 13A); Animal number 7, sera drawn 42 days post LVS vaccination (scarification) (FIGURE 13B); Animal number 36, sera drawn 42 days post LVS vaccination (subcutaneous) (FIGURE 13C); Animal number 26, sera drawn 63 days post LVS vaccination (subcutaneous) (FIGURE 13D); and Animal number 19, sera drawn 63 days post LVS vaccination (scarification) (FIGURE 13E).

FIGURE 14 shows 2D-Western blots of sera from LVS-vaccinated rabbits. Group 1 animals were subcutaneously vaccinated with LVS and challenged on day 42 with SCHU S4 (FIGURE 14A). Group 2 animals were vaccinated with LVS by scarification and challenged on day 42 with SCHU S4 (FIGURE 14B). Group 3 animals were sham-vaccinated by scarification (right) or subcutaneously (left, in box) with saline and sera drawn 42 days post vaccination (FIGURE 14C). For FIGURES 14A-C, boxed sera are paired, with the left hand blot probed with sera drawn pre-vaccination and the blot on the right probed with sera drawn 42 days post-vaccination; remaining sera (where applicable) are unpaired and were probed with sera drawn 42 days post-vaccination. Group 4 animals were subcutaneously vaccinated with LVS and challenged on day 63 with SCHU S4 (FIGURE 14D). Group 5 animals were vaccinated with LVS by scarification and challenged on day 63 with SCHU S4 (FIGURE 14E). For FIGURES 14D-E, sera are paired, with the left hand blot in each pair having been probed with sera drawn pre-vaccination, and the blot on the right probed with sera drawn 63 days post-vaccination. Group 6 animals were sham-vaccinated by scarification (top) or subcutaneously (bottom) with saline and sera drawn 63 days post vaccination (FIGURE 14F). Group 7 of the LVS-vaccinated rabbit study is shown in FIGURE 14G. These blots (FIGURES 14F-G) were probed with sera from control animals

FIGURE 15 is a matrix of immunoreactive proteins for LVS-vaccinated rabbits. Shading indicates the degree of observed immunoreactivity, from no observed immunoreactivity (□), to intense immunoreactivity (■). * denotes animals that survived aerosol challenge with SCHU S4.

FIGURE 16 shows characteristics of immunoreactive proteins identified from Western blotting with sera from LVS-vaccinated rabbits. FIGURE 16A is a bar chart representation of the frequency with which each immunoreactive protein was observed in the LVS-vaccinated rabbit

sera screened. Black fill indicates immunoreactive proteins observed to react with sera from rabbits LVS-vaccinated by scarification. Grey fill indicates immunoreactive proteins reacting with sera from rabbits vaccinated SC with LVS. FIGURE 16B is a bar chart showing total intensity of observed immunoreactivity for LVS-vaccinated rabbits. Animals are grouped by route of vaccination, and by day sera was collected post-vaccination. Asterisk (*) indicates animals that survived SCHU S4 challenge.

FIGURE 17 shows 2D Western blots probed with sera from mice vaccinated subcutaneously or intranasaly with LVS. BALB/c mice were immunised with LVS subcutaneously with lot 17 LVS, and sera drawn at 4 weeks post-vaccination (Mouse 1.1; FIGURE 17A), or intranasaly 4 weeks post-vaccination (Mouse 4.1; FIGURE 17B). Antigen was 100 µg SCHU S4 Δ*wbtI*, separated in the pH range 4-7. Primary sera were used in a 1:500 dilution.

FIGURE 18 shows 2D Western blots probed with sera from mice vaccinated subcutaneously with LVS or LVS Δ*IglC* mutant. Western blots probed with sera from sham-vaccinated BALB/c mice (FIGURE 18A), LVS-vaccinated BALB/c mice (FIGURE 18B), and BALB/c mice vaccinated with LVS Δ*IglC* mutant (FIGURE 18C). Sera were drawn at 4 weeks post-vaccination. Representative blots are shown. Antigen was 100 µg SCHU S4 Δ*wbtI*, separated in the pH range 4-7. Primary sera were used in a 1:500 dilution.

FIGURE 19 shows the characteristics of immunoreactive proteins in successful intradermal LVS vaccination with unsuccessful LVS Δ*iglC* vaccination of BALB/c mice. FIGURE 19A is a matrix of immunoreactive proteins, showing results of 2D Western blots probed with sera from mice vaccinated subcutaneously with LVS or LVS Δ*IglC* mutant. FIGURE 19B is a bar chart showing total observed intensity in immunoreactive spots for each serum screened. FIGURE 19C is a bar chart showing observed immunoreactivity for areas corresponding to the proteins SucB/RspA. FIGURE 19D is a bar chart showing observed immunoreactivity for areas corresponding to the protein peroxidase/catalase. FIGURE 19E is a bar chart showing observed immunoreactivity for areas corresponding to the protein GroEL. FIGURE 19F is a bar chart showing observed immunoreactivity for areas corresponding to the protein DnaK.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to markers of *Francisella tularensis* infection and uses thereof. More specifically, the invention relates to markers of *Francisella tularensis* infection and their uses as correlates of protection.

The present invention provides a set of biomarkers for tularaemia, selected from the group consisting of: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 50S ribosomal protein L7/L12, 30S ribosomal protein S1, DNA-directed RNA polymerase alpha subunit, Acetyl-CoA carboxylase, Outer membrane associated protein FopA, Peroxidase/catalase, Chaperone protein DnaK, Pyruvate dehydrogenase E2 component, Chaperone protein groEL, Hypothetical membrane protein, and any combination thereof.

By the term "biomarker", also referred to as "biological marker", it is meant a molecule that is used as an indicator of a particular state. In the context of the present application, the biomarker is an immunogenic protein, also referred to as "immunoreactive antigen" or "commonly reactive antigen", that is reactive with sera from subjects previously infected with or vaccinated against tularaemia.

Tularemia, or "tularaemia", refers to a spectrum of diseases caused by the Gram-negative bacterium *Francisella tularensis*. *F. tularensis* is pathogenic for many mammalian species including humans, primates, and rodents. Several subspecies exist, most notably the clinically relevant *tularensis* (Type A) and *holarctica* (Type B). Type A is associated with mortality rates of up to 60%, while Type B is associated with a less severe clinical manifestation and lower mortality rates.

The biomarkers of the present invention include dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 50S ribosomal protein L7/L12, 30S ribosomal protein S1, DNA-directed RNA polymerase alpha subunit, Acetyl-CoA carboxylase, Outer membrane associated protein, fopA, Peroxidase/catalase, Chaperone protein dnaK, Pyruvate dehydrogenase E2 component, Chaperone protein groEL, and Hypothetical membrane protein. Any combination of the aforementioned proteins is also included within the scope of the present invention. These proteins are described in Table 1.

Table 1. Downselected protein biomarkers of tularemia infection and vaccination. The locus tag of the proteins refers to the *Francisella tularensis* SCHU S4 genome sequence.

Locus tag	Protein Name	MW (kD)	pI	SEQ ID NO:
FTT_0077	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	57.5	5.83	1
FTT_0143	50S ribosomal protein L7/L12	12.8	4.62	2
FTT_0183	30S ribosomal protein S1	61.5	5.16	3
FTT_0350	DNA-directed RNA polymerase alpha subunit	35.3	4.93	4
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	16.4	5.00	5
FTT0583	Outer membrane associated protein, FopA	41.4	5.58	6
FTT_0721c	Peroxidase/catalase	82.5	5.37	7
FTT_1269c	Chaperone protein dnaK	69.21	4.88	8
FTT_1484c	Pyruvate dehydrogenase E2 component	67.3	4.77	9
FTT_1696c	Chaperone protein, groEL	57.3	4.96	10
FTT_1778c	Hypothetical membrane protein FTT_1778c	13.7	8.67	11

Each of the biomarkers as described above may independently be reactive with tularaemia-exposed sera at a frequency of about 30-100%. For example, each of the biomarkers may independently be reactive with sera at a frequency of about 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100%, or any frequency there between.

The present invention also provides a method of evaluating immunity against tularaemia in a subject, comprising:

a. contacting serum from the subject with the biomarkers of the present invention;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. determining the protection against tularaemia based on immunoreactivity to the biomarkers.

By the term "immunity", it is meant the ability or capability to avoid infection or disease or lessen its effects. In the context of the present invention, "immunity against tularaemia" indicates that the subject is protected against tularaemia. The particular aspect of immunity being evaluated by the method of the present invention is humoral immunity. As described above, humoral immunity, including specific antibody responses has been observed following natural *Francisella* infections or vaccination (Dennis et al, 2001; Saslaw & Carhart, 1961; Carlsson et al, 1979; Viljanen et al, 1983). Because subjects that have recovered from Types A and B *Francisella* infections rarely show signs of disease following a second exposure, those subjects represent a population that is immune to further challenge. Thus, evaluating immunity

of subjects against tularaemia based on antibody response may provide a direct correlation to overall immunity to tularaemia.

The subject in which the immunity against tularaemia is evaluated may be any suitable subject. The subject may be a mammal; for example, and without wishing to be limiting in any manner, the subject may be a human, a primate, a rodent (such as a rabbit or mouse), any other suitable mammalian subject. The subject may or may not have been exposed to tularaemia prior to evaluation of immunity.

The immunoreactivity of the serum to the selected biomarkers may be evaluated by any suitable method known in the art. For example, and without wishing to be limiting in any manner, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) Western blotting may be used to evaluate immunoreactivity of the biomarkers to the subject's serum; once proteins are isolated they may be identified by mass spectrometry. Other suitable methods known to those of skill in the art may also be used to evaluate immunoreactivity to the biomarkers. This could include the use of a *Francisella* proteome microarray (Eyles et al, 2007), an ELISA screening method, a dot-blot technique, or any other technique or method relying on antibody-antigen reactivity. Persons of skill in the art would be familiar with such techniques, which have been described in detail in the art.

In the method described above, a determination of protection against tularaemia can be made based on the immunoreactivity of the subject's serum to the biomarkers; in other words, a person of skill in the art can determine whether the subject is protected against tularaemia or not. Immunoreactivity to selected biomarkers of the present invention may be an indicator that the subject is protected against tularaemia; immunoreactivity to a combination of the biomarkers of the present invention is a good indication of protection. Immunoreactivity may be to one biomarker of the invention or to a combination of two, three, four, five, six, seven, eight, nine, ten or eleven biomarkers of the invention.

The present invention further provides a method of predicting vaccine efficacy, comprising:

a. contacting serum from a vaccinated model of tularaemia with the biomarkers of the present invention;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. correlating the immunoreactivity to immunoprotective status of the model; and

d. predicting vaccine efficacy in human based on the correlation of step c.

By "predicting vaccine efficacy", it is meant that the efficacy of a vaccine in conferring immunity to a human subject is predicted by the method presently described. The serum from a vaccinated model of tularaemia is obtained. The model of tularaemia may be a human or animal model; in a non-limiting example, the model of tularaemia may be an animal model such as, but not limited to a mouse model, rabbit model, primate model, or other suitable animal model that simulates a human with respect to its susceptibility or reaction to tularaemia. In one specific example, the animal model may be a mouse, rabbit, non-human primate, or human. The immunoreactivity of the serum from the model of tularaemia to the biomarkers of the present invention is then evaluated.

The immunoreactivity to the selected biomarkers may be evaluated by any method known in the art. For example, and without wishing to be limiting in any manner, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) Western blotting may be used to evaluate immunoreactivity of the biomarkers to the subject's serum; once proteins are isolated they may be identified by mass spectrometry. Other suitable methods known to those of skill in the art may also be used to evaluate immunoreactivity to the biomarkers, including a *Francisella* proteome microarray (Eyles, et al, 2007), an ELISA screening method, a dot-blot technique, or any other technique or method relying on antibody-antigen reactivity.

The immunoreactivity of the serum from the model of tularaemia is then correlated to the immunoprotective status of the model. The immunoprotective status refers to whether the model is protected against tularaemia or not; this may be determined by any suitable method known in the art, for example, but not limited to subsequent exposure to tularaemia or challenge with an appropriate tularaemia strain.

Prediction of the vaccine efficacy in a human may be made based on the correlation between immunoreactivity and immunoprotective status as described above. The pattern of immunoreactivity in the model is observed and compared to the immunoreactivity in a vaccinated human. If the markers of the present invention show a similar pattern of immunoreactivity in the human as in the model, then the skilled person can extrapolate that the human will have the same immunoprotective status as the model. For example, and without wishing to be limiting in any manner, if the model is protected and the human's markers show the same pattern of immunoreactivity as the model's, then it may be extrapolated that the human is protected.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Sera preparations

Four distinct collections of human sera were used in Examples 2 and 3, including human tularemia patients (two separate groups), LVS-vaccinated laboratory personnel, and clinical trial subjects immunized with LVS. The details of each sera screened are shown in Table 2 and summarized briefly below.

The Type B convalescent sera were obtained from patients diagnosed with tularemia in a region of Sweden, where the disease is considered endemic. Control sera were obtained from individuals with no history of tularemia or a tularemia-like disease. In total, sera were obtained from 12 tularemia patients and 3 healthy individuals with no history of tularemia. Since Type A strains are endemic to North America only, the Swedish patients were exclusively infected with type B strains. The route of infection for the majority of these patients was intradermal.

The Type A convalescent sera were obtained from a subset of 59 subjects with presumed or confirmed cases of tularemia reported on Martha's Vineyard between 2000 and 2006. Approximately 60 % were thought to be due to inhalation of the bacterium (Matyas et al, 2007; Feldman et al, 2003). Sera from the first physician visit were available from 12 confirmed Type A tularemia patients.

Two sets of sera from separate human LVS vaccinations were obtained. The first set, in which at-risk laboratory workers in Sweden were immunized with LVS NDBR101 Lot 11, was comprised of 5 sets of paired pre- and post-vaccination samples and an additional 3 post-vaccination serum samples. NDBR lot 11 was prepared as per the vial instructions. Briefly, the vaccine preparation was reconstituted in 2.0 ml of water to give a concentration of 2.5×10^9 CFU /ml. A droplet of approximately 20 µl (containing ~ 5×10^7 CFU) was administered by scarification using a bifurcated needle that was used to puncture the skin.

The second human vaccinee serum set was from subjects vaccinated with a recently manufactured lot of LVS (DVC lot 17) and was obtained from a Phase I clinical trial carried out at the Baylor College of Medicine, Houston, TX. The vaccine used was manufactured at Cambrex Bio Science, Baltimore, MD, under contract with Dynport Vaccine Company LLC (DVC). Vaccine was administered as described previously (El Sahly et al, 2009). Briefly, the lyophilized vaccine was reconstituted with 0.25 ml of sterile water for injection yielding a

vaccine concentration of 1.6 10^9 CFU/ml. The study design and administration of the vaccine was described in detail previously (El Sahly et al, 2009), with dosages of 10^3, 10^5, 10^7 and 10^9 CFU/ml administered by scarification with a bifurcated needle. Five paired sera (pre- and 42 days post-vaccination) and three unpaired sera (post-vaccination) were provided.

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Table 2. Summary of sera used in screening.

Type	Sample reference number	Date of diagnosis	Date or length of time post infection/vaccination serum drawn	Route	Pairing	Origin
Type B convalescent	1651	Unknown	42 months		NA	Sweden
Type B convalescent	1653	Unknown	17 months		NA	Sweden
Type B convalescent	1657	Unknown	44 months		NA	Sweden
Type B convalescent	1661	Unknown	18 months		NA	Sweden
Type B convalescent	1663	Unknown	18 months		NA	Sweden
Type B convalescent	1671	Unknown	18 months		NA	Sweden
Type B convalescent	1673	Unknown	18 months		NA	Sweden
Type B convalescent	1679	Unknown	42 months		NA	Sweden
Type B convalescent	1683	Unknown	18 months		NA	Sweden
Type B convalescent	1687	Unknown	17 months		NA	Sweden
Type B convalescent	1691	Unknown	30 months		NA	Sweden
Type B convalescent	1693	Unknown	17 months		NA	Sweden
Type A convalescent	f0703	Unknown	08/02/ 2006		NA	NE, USA
Type A convalescent	f0709	2005	22/02/ 2006		NA	NE, USA
Type A convalescent	f0711	Unknown	24/05/ 2006		NA	NE, USA
Type A convalescent	f0715	Unknown	14/03/ 2006		NA	NE, USA
Type A convalescent	f0722	Unknown	04/04/ 2005		NA	NE, USA
Type A convalescent	f0723	Unknown	04/04/ 2005		NA	NE, USA
Type A convalescent	f0753	2006	21/11/ 2007		NA	NE, USA
Type A convalescent	f0754	2007	21/11/ 2007		NA	NE, USA
Type A convalescent	f0756	2006	21/12/2007		NA	NE, USA
Type A convalescent	f0757	2006	21/12/2007		NA	NE, USA
Type A convalescent	f0758	2007	05/01/ 2008		NA	NE, USA
Type A convalescent	f0759	1999	16/01/ 2008		NA	NE, USA
NDBR Lot 11 Vaccinee	201	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	202	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	203	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	204	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	205	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	206	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	207	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Vaccinee	208	NA	42 days	Scarification	NA	Sweden
NDBR Lot 11 Control	111	NA	Control	Scarification	NA	Sweden
NDBR Lot 11 Control	112	NA	Control	Scarification	NA	Sweden
NDBR Lot 11 Control	113	NA	Control	Scarification	NA	Sweden
NDBR Lot 11 Control	114	NA	Control	Scarification	NA	Sweden
NDBR Lot 11 Control	115	NA	Control	Scarification	NA	Sweden
DVC Lot 17 Vaccinee	200162501	NA	2	Scarification	02855	USA

				(10^9 cfu/mL)		
DVC Lot 17 Vaccinee	200161436	NA	10	Scarification (10^9 cfu/mL)	02855	USA
DVC Lot 17 Vaccinee	200139305	NA	2	Scarification (10^5 cfu/mL)	02816	USA
DVC Lot 17 Vaccinee	200162408	NA	10	Scarification (10^5 cfu/mL)	02816	USA
DVC Lot 17 Vaccinee	200162457	NA	02	Scarification (10^9 cfu/mL)	02858	USA
DVC Lot 17 Vaccinee	200161482	NA	10	Scarification (10^9 cfu/mL)	02858	USA
DVC Lot 17 Vaccinee	200161191	NA	10	Scarification (10^7 cfu/mL)	02840	USA
DVC Lot 17 Vaccinee	200162938	NA	10	Scarification (10^7 cfu/mL)	02838	USA
DVC Lot 17 Vaccinee	200139359	NA	02	Scarification (10^5 cfu/mL)	02807	USA
DVC Lot 17 Vaccinee	200162272	NA	10	Scarification (10^5 cfu/mL)	02807	USA
DVC Lot 17 Vaccinee	200139603	NA	02	Scarification (10^7 cfu/mL)	02832	USA
DVC Lot 17 Vaccinee	200162794	NA	10	Scarification (10^7 cfu/mL)	02832	USA
DVC Lot 17 Vaccinee	200208090	NA	10	Scarification (10^3 cfu/mL)	02875	USA

NA – not applicable

Example 2: Two-dimensional polyacrylamide gel electrophoresis Western blotting

The four collections of human sera described in Example 1 were used in two-dimensional polyacrylamide gel electrophoresis (2D PAGE) Western blotting experiments in order to determine the repertoire of immunoreactive proteins for each serum sample. Briefly, the proteins of a bacterial cell lysate were separated in two dimensions – by protein isoelectric point then by protein molecular mass using 2D-PAGE, as described in Twine et al (2005; 2010). Resolved proteins were then transferred to nitrocellullose membrane by electroblotting and the membrane was subsequently incubated with serum from Example 1. Antibodies in the serum recognised their cognate antigen on the membrane and this antibody binding was subsequently detected, generating a pattern of immunoreactive proteins.

Francisella tularensis ΔwbtI, a mutant strain lacking the O-antigen, was used as the protein antigen in blotting experiments. Briefly, bacteria were grown in modified Cysteine Heart agar (CHA) for 24–36 h at 37°C within a BioSafety (BS) Level 3 containment facility. Plate grown bacteria were harvested directly into lysis buffer (5 M Urea, 2M Thiourea, 4 % CHAPS, 0.5 % ASB-14), as described in earlier work (Twine et al, 2005), in order to solublise bacterial proteins. A portion (10 %) of all cell lysates were plated on CHA and checked for sterility after incubation at 37°C for 36 hours before release of bacteria from the BS Level 3 facility. The resulting proteins in the lysate were quantified using a Bradford protein assay.

The lysates were separated using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) Western blotting. Briefly, proteins were separated in the first dimension by isoelectric point, with 100 ug of bacterial proteins, dissolved in Immobilised pH gradient solutions (Biolytes (3-10): Biolytes stock (3-10, Biorad) and 10µg Orange G). Immobilised pH gradient (IPG) strips were rehydrated with bacterial proteins, as described in the manufacturer's instructions (Biorad, Hercules, CA). Isoelectric focussing was carried out using a Protean Cell (Biorad, Hercules, CA) with the following 24 hour programme: 200 V for 1 hour, 500 V for 1 hour, ramp to 5000 V over 5 hours, focus to 80,000 Vh at 5000 V, at 20 °C, for a cumulative total of 95,000Vh. Previous work with murine sera showed no proteins with a pI <4 or >7 reactive with sera from LVS immunized BALB/c or C57/BL6 mice (Twine et al, 2010; Twine et al, 2006); therefore, the present analyses were confined to pH 4-7. Second dimension separations were carried out using 10 % SDS-PAGE. IPG strips were equilibrated with 2mL DTT solution (0.05 g DTT, 0.1 g SDS, 0.68mL 1M Tris HCl, pH 8.8, 3.6 g urea, 3g glycerol, in MQ water up to 5 mL), at room temperature for 20 minutes, followed by equilibration with 2mL iodoacetamide solution (0.2 g iodoacetamide, 0.1 g SDS, 0.68mL 1M Tris HCl, pH 8.8, 3.6 g urea, 3g glycerol, in 5 mL), at room temperature for 20 minutes.

Immunoblotting was carried out according to previously published methods (Mansfield, 1995; Twine et al, 2010). The human sera described in Example 1 were used at a dilution of 1:500, and secondary antibody (horseradish peroxidase labelled) was used as per the manufacturer's instructions. Immunoreactivity on nitrocellulose membranes was visualised using a commercially available ECL (GE Healthcare, Baie d'Urfe, Canada). Blots were developed by 30s or 1 minute exposure to Kodak Biomax Scientific imaging film. Developed blots were aligned with either images of the protein-stained nitrocellulose membrane, after protein transfer, or protein stained 2D-PAGE. Protein spots observed to be immunoreactive were identified as described in Example 3.

The complete series of Western blots are shown in Figures 1 to 4. Specifically, Figure 1 shows Western blots probed with sera from Type B patients; Figure 2 shows Western blots probed with sera from Type A patients; Figure 3 shows Western blots probed with sera from LVS vaccinees (Umea); and Figure 4 shows Western blots probed with sera from LVS vaccinees (Baylor). Figure 5 is a comparison of representative 2D Western blots of Figures 1 to 4.

Example 3: Identification of immunoreactive proteins

Immunoreactive proteins observed by 2D Western blotting in Example 2 were identified by tryptic digest and mass spectrometry.

Protein spots corresponding to areas of immunoreactivity on Western blots were excised from equivalent protein stained 2D-PAGE gels and tryptically digested manually, as described previously (Twine et al, 2010; Twine et al, 2006). The in-gel digests were analyzed by nano-liquid chromatography-MS/MS as described previously (Twine et al, 2010). The peak list files of MS^2 spectra of the excised protein spots were searched against the translated SCHU S4 genome sequence using the MASCOT™ search engine (version 2.2.03) (Matrix Science, London, UK) for protein identification, as described in earlier work (Twine et al, 2010).

A total of 31 immunoreactive proteins were identified from Type B patients (Table 3, Figure 6). A single protein, Chaperonin GroEL (FTT_1696), was immunoreactive with all sera from all 12 patients, but with none of the control sera. The protein dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077) was immunoreactive with 11 of the 12 patient sera screened but none of the control sera. Further to this, the proteins 50S ribosomal protein L1/L12 (FTT_0143), hypothetical membrane protein (FTT_1778c), and acetyl CoA carboxylase (FTT_0472) was immunoreactive with 8 or more patient sera screened. The proteins FTT_1778c and FTT_0143 focused to discrete spots on 2D-PAGE within close proximity of one another. Where immunoreactivity of these proteins was intense, it was not always possible to discern which individual protein was immunoreactive, or to measure the intensity of immunoreactivity of the individual proteins. In these cases, it was indicated that both proteins were immunoreactive and the overall intensity of immunoreactivity reported. These data are also represented visually as a matrix of immunoreactive proteins, in Figure 7A. The shading indicates the comparative intensity of the observed immunoreactivity for each spot, as measured by densitometry, with darker shading denoting more intense immunoreactivity.

Table 3. A summary of proteins reactive with sera from human tularemia patient (Type A and B) or LVS vaccinees

			Study				
Locus tag	**GenInfo Identifier**	**Protein Name**	**Vaccine NDBR lot 11 LVS (n=8)**	**Vaccine DVC lot 17 LVS (n=8)**	**TypeB (n=12)**	**TypeA (n=12)**	**Previously reported**
FTT_0060	56707239	ATP synthase subunit B	-	-	5	1	(1), (2)
FTT_0062	56707241	ATP synthase subunit A	1	-	5	1	(2),(3),(4)
FTT_0064	56707243	F0F1 ATP synthase subunit beta	-	-	6	-	(3),(5)
FTT_0077	56707256	Dihydrolipoamide succinyltransferase component of 2-	8	7	11	8	(1),(2),(3),(4), (6)(7)

		oxoglutarate dehydrogenase complex					
FTT_0087	56707265	Aconitate hydratase	-	1	4	-	(3),(6),(7)
FTT_0137	56707307	Elongation factor Tu (EF-Tu)			1	4	(2),(3),(4),(6),(8)
FTT_1486c	56708525	Hypothetical lipoprotein	-	-	1	1	
FTT_0143	56707313	50S ribosomal protein L7/L12	8	6	8	10	(2),(4),(6),(8)
FTT_0183c	56707348	30S ribosomal protein S1	7	5	7	-	(2),(3),(4)
FTT_0188	56603845	Cell division protein	-	-	1	4	(2),(3),(4),(6)
FTT_0191	56707356	Peptide chain release factor 2		-	-	1	
FTT_0196c	56707361	Glutamine synthetase	4	2	-	-	(1), (2)
FTT_0307	56707460	Glutamyl-tRNA synthetase			1	-	
FTT_0323	56707476	Elongation factor G (EF-G)	1	1	3	2	(2),(3),(4)
FTT_0350	56707503	DNA-directed RNA polymerase alpha subunit	5	3	4	-	, (2),(3)
FTT_0373c	56707524	Nucleoside diphosphate kinase	-	-	-	1	(2),(8)
FTT_0407	56707556	Aminomethyltransferase	1	-	-	-	(2)
FTT_0408	56707557	Glycine cleavage system H protein	2	-	3	-	
FTT_0472	56707614	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	6	3	8	7	(1), (3), (4),(8)
FTT_0504c	56707643	Succinyl-CoA synthetase subunit beta	2	-	-	-	(7)
FTT_0583	56707711	Outer membrane associated protein, fopA	8	3	1	6	(1), (3),(4),(6)
FTT_0673c	56708418	Hypothetical protein FTT0673c	-	-	3	-	
FTT_0715	56707834	Chitinase family 18 protein	-	-	1	-	(2),(3),(4)
FTT_0721c	56707839	Peroxidase/catalase	5	5	6	7	(2),(3),(4),(5),(6),(7),(8)
FTT_0726c	56707843	Glycerophosphoryl diester phosphodiesterase family protein	6	3	-	-	(6)
FTT_0817	56707928	Threonyl-tRNA synthetase	1	-	-	-	(2),(4)
FTT_0893	56707994	Phosphoribosylaminoimidazole synthetase	5	2	-	-	
FTT_1103	56708183	Hypothetical lipoprotein	-	-	1	-	(1),(2),(3),(4),(5),(6)
FTT_1269c	56708329	Chaperone protein dnaK	5	7	5	8	(1),(2),(3),(4),(5),(6),(7),(8)
FTT_1281c	56707258	Sigma-54 modulation protein	-	-	1	-	(2),(8)
FTT_1314c	56708371	Type IV pili fiber building block protein	2	-	4	-	(1), (2)
FTT_1352	56708408	Hypothetical protein	-	4	2	-	

		FTT1352					
FTT_1357c	56708413	Intracellular growth locus, subunit C	3	3	-	-	(2),(5)
FTT_1365c	56708418	Fructose-bisphosphate aldolase	-	-	3	-	
FTT_1368c	56708421	Glyceraldehyde-3-phosphate dehydrogenase	-	-	3	-	(2),(8)
FTT_1373	56708426	3-oxoacyl-[acyl carrier protein] synthase III	-	-	4	2	(3)
FTT_1483c	56708522	Dihydrolipoamide dehydrogenase	-	-	1	-	(6)
FTT_1484c	56605015	Pyruvate dehydrogenase, E2 component	4	3	3	10	(6),(7) (1), (3)
FTT_1485c	56708522	Pyruvate dehydrogenase subunit E1	-	-	-	2	(7)
FTT_1696	56708705	Chaperone protein, groEL	4	6	12	9	(1), (3), (4), (5),(6)
FTT_1778c	56708771	Hypothetical membrane protein FTT1778c	8	6	8	5	(1), (3),(4)

(1) Eyles et al, 2007; (2) Titball et al, 2007; (3) Twine et al, 2010; (4) Twine et al, 2006; (5) Huntley et al, 2007; (6) Janovska et al, 2007a; (7) Janovska et al, 2007b; (8) Havlasova et al, 2005)

A total of 19 proteins were identified as immunoreactive with sera from one or more of the Type A tularemia patients. No single protein was observed to be immunoreactive with all Type A tularemia patient sera screened, although the proteins pyruvate dehydrogenase E2 (FTT_1484c) and ribosomal protein L7/L12 (FTT_0143) were observed to be reactive with 10 of the 12 sera studied. In addition, the proteins dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase (FTT_0077) and chaperonin protein DnaK (FTT_1269c) were reactive with 8 of the total 12 sera.

Across all eight post-vaccination serum samples from LVS vaccinated Swedish laboratory workers (LVS NDBR101 Lot 11) screened, a total of 22 immunoreactive proteins were identified (Table 3, Figure 7). Four proteins were observed to be immunoreactive to some degree with all of the post-vaccination sera: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077), 50S ribosomal protein L7/L12 (FTT_0143), outer membrane protein FopA (FTT_0583) and hypothetical protein (FTT_1778c). Of the pre-vaccination sera, one individual showed no detectable immunoreactivity. The remaining four sera showed weak immunoreactivity with the proteins 30S ribosomal protein S1 (FTT_0183c), 50S ribosomal protein L7/L12 (FTT_0143) and hypothetical protein (FTT_1778c).

Blots probed with sera from humans vaccinated with the new CGMP formulation of LVS (DVC lot 17) showed immunoreactivity with a total of 18 proteins (Table 3). For the post-vaccination

sera, no single protein was reactive with all sera; however, the proteins dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077) and chaperonin dnaK (FTT_1269c) were reactive with seven of the eight post-LVS vaccination sera. It is also interesting to note that the outer membrane protein FopA (FTT_0583), was reactive with only three post-vaccination sera. The pre-vaccination sera showed no reactivity (one subject) or weak reactivity towards the proteins dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077), 30S ribosomal protein S1 (FTT_0183c), catalase (FTT_0721c), 50S ribosomal protein L7/L12 (FTT_0143), and hypothetical protein (FTT_1778c). The escalating vaccine dose did not influence the repertoire of immunoreactive proteins, with the exception of post-vaccination sera from one subject (#200162938), vaccinated with 10^7 CFU, showed no detectable immunoreactivity.

Figure 7 shows the repertoire of immunoreactive proteins catalogued for each serum sample screened. This figure also illustrates the relative intensity of each observed immunoreactive spot and shows the marked heterogeneity in the immunoreactivity. The bar chart below the matrix of immunoreactive proteins in Figure 7A shows the sum of the comparative intensity values for the identified immunoreactive proteins. With the exception of the NDBR lot 11 LVS vaccinees, the mean total relative intensity in identified immunoreactive spots was similar for each group of sera screened. One subject from each of NDBR lot 11 LVS vaccinees, Type A and Type B tularemia patients, showed a markedly higher total intensity of immunoreactive proteins. In contrast, two other sera drawn 42 days post-vaccination with 10^5 CFU DVC lot 17 LVS, also showed comparatively low immunoreactivity. The greatest total relative intensity of identified immunoreactive proteins was observed for the vaccination dose of 10^9 CFU.

The properties of the reactive proteins were examined according to computationally predicted features to determine whether a particular type of protein was over-represented. First, the PSORT1b algorithm was used, an algothrim that predicts the subcellular localization of proteins based upon amino acid sequences (Gardy et al, 2005). Figure 7B shows graphically that the vast majority of identified immunoreactive proteins were predicted to be cytoplasmic in location (56 %), with 36 % of the proteins of unknown location (36%). The remaining proteins were localized to various locations, including the outer membrane and periplasm. Secondly, the identified proteins were classified according to the Clusters of Orthologous Groups (http://www.ncbi.nlm.nih.gov/COG/; Tatusov et al, 1997; Tatusov et al, 2003) and the identified proteins were classified according to predicted function. Figure 7C shows 23% of the identified proteins were predicted to be involved in energy production and conversion (COG C), 20% predicted to be involved in translation, and 15% to be of unknown function.

Figure 8 shows a graphical representation of the frequency with which each immunoreactive protein was identified, regardless of experimental group. Of note, the protein dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077) was reactive with 70% of all sera screened. By contrast, the outer membrane protein FopA was observed to be reactive with sera from all LVS NDBR lot 11 vaccinees, but less than half of the subjects from other groups. From this graph, and the matrix of immunoreactive proteins in Figure 7A, 11 proteins were identified as commonly reactive antigens, reactive with both patient and vaccinee sera, with a minimum frequency of 30%. These included dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FT_0077), 50S ribosomal protein L7/L12 (FTT_0143), 30S ribosomal protein S1 (FTT_0183), DNA-directed RNA polymerase alpha subunit (FTT_0350), Acetyl-CoA carboxylase (FTT0472), Outer membrane associated protein FopA (FTT_0583), Peroxidase/catalase FTT_0721c), Chaperone protein DnaK (FTT_1269c), Pyruvate dehydrogenase E2 component (FTT_1484c), Chaperone protein groEL (FTT1696), Hypothetical membrane protein (FTT_1778c).

It was observed that the repertoire of proteins reactive with sera from individuals recovering from natural Type A and B infection showed a great deal of overlap, as shown in Figure 7. This is interesting, given that the most common route of infection for Type A *Francisella* in the Martha's Vineyard patients is by inhalation (Matyas et al, 2007), whereas the most common route of infection in the European Type B-infected group is presumed to be arthropod-borne intradermal (Sjostedt, 2007). Therefore, the antibody repertoire generated in response to natural infection with Type A or B strains of *Francisella* has a large degree of similarity, despite differences in bacterial strains and routes of infection. Subtle differences in the immunoproteomic profiles when screened against the same *Francisella* antigen, however, were also observed. For example, 80% of Type A tularemia convalescent sera showed some degree of reactivity towards the protein pyruvate dehydrogenase E2 component. By contrast, sera from Type B tularemia patients showed reactivity towards the same protein in less than 50% of patients analysed. In addition, sera from Type B tularemia patients showed reactivity with a greater repertoire of proteins, with a total of thirty-one proteins observed to be reactive with sera from Type B patients, compared with eighteen with sera from Type A patients. It is difficult, however, to draw conclusions regarding the significance of these observations, given that the exact date and route of infection for each patient is unknown, as is the longevity of the circulating anti-*Francisella* antibodies (a majority of patients lacked demonstrable antibody titres 25 years after infection; Ericsson et al, 1994). In relation to this, a recent study reported the repertoire of immunoreactive proteins in the sera of a laboratory worker, accidentally infected with Type A *Francisella*, did not markedly change over a period of 16 years

(Janovska, 2007b). The one exception was a single immunoreactive protein that was not observed to be reactive at later time points after infection. Of note, 5 of the 10 identified immunoreactive proteins described here were also shown in an earlier study to be reactive with sera from patients recovering from Type A infections (Janovska, 2007b).

The reactive antigens were not evenly distributed across the proteome. For example, in terms of predicted subcellular location, cytoplasmic proteins were by far over-represented. Without wishing to be bound by theory, this may result from a bias introduced by the gel-based immunoproteomics approach, which is known to have limited capability to resolve very large, small or hydrophobic proteins. A recently developed alternative is the proteome chip, where cell-free expressed proteins immobilized on microarray style chips are probed with immune sera (Sundaresh et al, 2007; Eyles et al, 2007). This approach was used to screen sera from LVS vaccinated mice and did not show the same bias towards cytoplasmic proteins. However, this approach also has limitations, including potential improper protein folding and lack of post-translational modifications of many proteins (Felgner et al, 2009). For many laboratories, the cost of this proteome chip approach can also be prohibitive. Aside from possible limitations of any experimental approach, and without wishing to be bound by theory, it may also be that certain functional categories of proteins are selectively recognized by the human immune system. The characteristics of the identified antigenic proteins that allow them to be selectively recognized by the immune system have not been identified.

No single protein was seen to be immunoreactive with all sera screened. However, eleven proteins were observed to react with the majority of sera screened and are denoted 'commonly reactive proteins'. Together, reactivity with a combination of these proteins may be predictive of vaccinees' protection from challenge with virulent strains. Since the incidence of re-infection is extremely low, patients recovering from either Type A or B tularemia may be assumed to be protected against re-infection. Thus, antigens common to patient and vaccinee sera are more likely to serve as potential correlates of protection. Therefore, the immunoreactive proteins indentified herein may be used to correlate with the protective status of the host.

Due to the inability to conduct vaccine efficacy studies in humans, development and evaluation of vaccine candidates will rely on animal models, bridging efficacy to humans based on correlates of protection. Several immunoproteomics studies of the murine humoral response to LVS vaccination have been reported (Eyles et al, 2007) and eleven of the present antigens have been reported previously in mice (Table 3). As other animal models of tularemia are developed and characterized, there exist opportunities to directly correlate the profile of immunoreactive proteins generated by LVS vaccination with the protective status of the host animal.

In addition to increasing understanding of the humoral immune response to primary pneumonic tularemia, systemic tularemia and tularemia vaccination, the identified immunoreactive proteins may be used to design and develop protein subunit based tularemia vaccine candidates.

Example 4: Immunoproteomics of Non-human primate model of tularemia

An important bridge between small animal models of tularemia and humans is the characterization of tularemia in non-human primates. The non-human primate (NHP) model of tularemia used in this Example was developed by Battelle Biomedical Research Centre (BBRC) in Rhesus macaques.

A large scale LVS vaccination of non-human primate was carried out by BBRC (Battelle Study No. 985-G006023: *Francisella tularensis* LVS Vaccination Efficacy Against a *F. tularensis* SCHU S4 Aerosol Exposure in Rhesus Macaques). This was a large scale LVS efficacy study comprised 40 Rhesus Macaques. As shown in Table 4, six groups of animals were vaccinated with either saline or LVS, either subcutaneously (SC) or by scarification. The vaccinating dose of LVS was 8 $x10^7$ cfu. Groups 1, 3 and 4 were then challenged on day 35 post vaccination with 2.5 x 10^6 cfu of SCHU S4 by aerosol. Animals in groups 2, 5 and 6 were challenged with the same dose of SCHU S4 by aerosol on day 63 post vaccination. Sera collected from animals pre-vaccination and prior to challenge, prior to challenge, were screened by 2D Western blotting (see Example 2).

Table 4. Non-human primate LVS vaccination efficacy study design and mortality data. In each case, challenge dose was 2.5 x 10^6 cfu of SCHU S4 by aerosol.

Group	NHP per group	Vaccine dose	Vaccination route	Challenge day (post vaccination)	Number of survivors
1	4	Saline	2 by SC 2 by Scar	35	0
2	4	Saline	2 by SC 2 by Scar	63	0
3	10	8 x 10^7 cfu	Scar	35	7
4	10	8 x 10^7 cfu	SC	35	8
5	10	8 x 10^7 cfu	Scar	63	8
6	10	8 x 10^7 cfu	SC	63	7

In addition, Table 4 indicates the proportion of animals that survived challenge, with none of the saline-vaccinated animals surviving. In comparison, 70-80 % of the LVS-vaccinated animals survived beyond 35 days post challenge.

2D-PAGE and Western blotting was performed as described in Example 2. Representative 2D Western blots are shown in Figure 9; full results are shown in Figure 10. Figure 9A is representative of Western blots probed with sera collected from animals pre-LVS vaccination. All sera collected from animals at either 28 or 56 days post LVS vaccination showed a marked increase in the number of regions of immunoreactivity and the intensity of immunoreactivity observed.

Immunoreactive areas observed on Western blots were aligned, where possible, with corresponding regions of protein staining on 2D-PAGE. The immunoreactive proteins were identified (see Example 3) and are shown in the matrix of immunoreactive proteins, in Figure 11; due to the number of sera screened and space constraints, only data from post-vaccination sera are shown. Animals that survived subsequent SCHU S4 challenge are indicated in this figure by greyscale shading of the box indicating animal number, versus white for animals that succumbed to challenge. Blots probed with pre-vaccination sera showed minimal or no immunoreactivity (data not shown).

In general, the repertoire of immunoreactive proteins observed to be reactive with the post-vaccination NHP sera showed a great deal of overlap with the immunoreactive proteins identified in Example 3. Of particular note, outer membrane protein FopA (FTT_0583) and DNA directed RNA polymerase A1 (FTT_0350), which focus to the same protein spots on 2D-PAGE, were reactive with all post-vaccination sera. In addition, the proteins FTT_0077 and FTT_0183 were reactive with almost all of the sera screened. There appeared no clear pattern of immunoreactivity that distinguished animals vaccinated via different routes, nor survivors versus non-survivors of SCHU S4 challenge.

Figure 12 shows a plot of total observed protein immunoreactivity from Western blots of sera from LVS-vaccinated animals. Within the chart, animals are grouped by route of vaccination and date of serum collection, in order to determine whether a relationship exists between total observed protein immunoreactivity and survivors of SCHU S4 challenge. The intensity of total immunoreactivity showed a large variation between individual animals, with no clear distinction between vaccinated animals that survived challenge and or those that succumbed to challenge with SCHU S4.

Example 5: Immunoproteomics in rabbit model of tularemia

The rabbit model of tularemia used in this Example was developed by the Midwest Research Institute (MRI) in New Zealand white rabbits.

Serum samples were provided by the Midwest Research Institute (MRI Project No. 110645.1.006: Evaluation of DVC *F. tularensis* Live Vaccine Strain (LVS) Efficacy in the New Zealand White Rabbit against Aerosol Challenge with DVC *F. tularensis* SCHU S4). A comprehensive characterization of LVS vaccination in New Zealand White Rabbits was established, with two routes of vaccination (Scarification and SC) and challenge dates either 42 or 63 days post-LVS vaccination. The study design is summarized in Table 5.

In total, 84 sera were provided, comprising of 64 sera from LVS-vaccinated rabbits (paired pre- and post-vaccination sera), eight sera from sham-vaccinated animals and sera from two control animals. The post-LVS vaccination sera were drawn either 42 days or 63 days post vaccination.

Table 5. Evaluation of DVC *F. tularensis* Live Vaccine Strain (LVS) Efficacy in the New Zealand White Rabbit against Aerosol Challenge with DVC *F. tularensis* SCHU S4 Study design.

Group	# Rabbits	Vaccine	Dose (cfu)	Route	Challenge day	Challenge dose (SCHU S4)
1	8	LVS	$8x10^7$	Scarification	42	10^3
2	8	LVS	$8x10^7$	SC	42	10^3
3	4	Saline	NA	Scar/SC	42	10^3
4	8	LVS	$8x10^7$	Scarification	63	10^3
5	8	LVS	$8x10^7$	SC	63	10^3
6	4	Saline	NA	Scar/SC	63	10^3
7	2	None	NA	NA	NA	NA

Pre- and post- vaccination sera from each animal in the study groups listed in Table 5 were screened by 2D Western blotting as described in Example 2. Figure 13 shows representative blots from this series, showing a blot probed with serum drawn pre-LVS vaccination (Figure 13A), and 42 or 63 post LVS vaccination by either SC or scarification (Figure 13B-E). Full results of Western blots are shown in Figure 14. The Western blot probed with sera drawn pre-vaccination showed only weak immunoreactivity towards a small number of proteins. This was typical for all pre-vaccination sera screened. In addition, blots probed with sera from

sham-vaccinated or control animals showed few, if any, weakly reactive areas of immunoreactivity. In contrast, all Western blots probed with sera drawn from animals post-LVS vaccination showed a marked increased in the observed total observed immunoreactivity and the number of immunoreactive proteins recognized.

Western blot images were aligned with equivalent protein stained gels, and regions that aligned with areas of protein staining were excised and the corresponding proteins identified by nLC-MS/MS of their tryptic digests (see Example 3). From this, a total of 25 immunoreactive proteins were identified. As in previous Examples, some intense areas of immunoreactivity corresponded to more than one protein spot (e.g., FTT_0077/FTT_0183), several immunoreactive protein spots focussed to multiple areas on 2DE (e.g., FTT_0583) and in some instances immunoreactivity did not correspond with detectable protein staining. In the latter case, it is important to note that immunoreactivity is not necessarily proportional to protein concentration, and the corresponding immunoreactive protein may be beyond the limits of silver staining detection in the 2D-PAGE approach.

The identified immunoreactive proteins are shown as a matrix of immunoreactive proteins in Figure 15, and the details of mass spectrometry identification are summarized in Example 2. Due to the large number of sera screened, data from pre-vaccination sera, sham-vaccinated animals and control animals have been omitted. As indicated by an asterisk in Figure 15, only six animals survived aerosol challenge with SCHU S4 (MRI Project No. 110645.1.006: Evaluation of DVC *F. tularensis* Live Vaccine Strain (LVS) Efficacy in the New Zealand White Rabbit against Aerosol Challenge with DVC *F. tularensis* SCHU S4). Of the six animals, one from each of the SC vaccination groups survived SCHU S4 challenge. A further three animals vaccinated by scarification survived challenge at 42 days post-vaccination, and a single animal also vaccinated by scarification, survived challenge at 63 days post vaccination.

The number of immunoreactive proteins identified for each LVS-vaccinated animal varied with, on average, ten immunoreactive proteins identified from blots probed with sera from both SC LVS-vaccinated rabbits drawn at both 42 and 63 days post-vaccination. By comparison, the average number of identified immunoreactive proteins from blot probed with sera from rabbits vaccinated by scarification was six (42 days post-vaccination) and seven (63 days post-vaccination). From the matrix of immunoreactive proteins, it can be seen that some proteins are immunoreactive with many of the post-vaccination sera screened. In order to gauge how frequently certain proteins were recognized by immune sera, the frequency with which each immunoreactive protein was observed was plotted as a bar chart (Figure 16). Of note, the proteins FTT_1778c/FTT_0143, FTT_1696, FTT_1539c, FTT_1374, FTT_0721c, and FTT_0583/FTT_0350 were reactive with more than half of the post-LVS vaccination sera

screened in this study. In particular, seven proteins, FTT_0715, FTT_1357c, FTT_1539c, FTT_1445, FTT_1338c, FTT_1695 and FTT_1155c were only observed to be reactive with sera drawn from subcutaneously vaccinated rabbits. Other proteins such as Catalase/peroxidase (FTT_0721c) were observed to be reactive with all sera from rabbits vaccinated SC and with only half of the sera drawn from rabbits vaccinated by scarification. Interestingly, Outer membrane protein FopA (FTT_0583) and DNA directed RNA polymerase (FTT_0183) were observed to be reactive with all post-LVS vaccination sera screened, regardless of the route of LVS vaccination or day post-vaccination that sera was drawn.

In addition to variation in the repertoire of immunoreactive proteins, sera from LVS-vaccinated rabbits showed variation in the total observed intensity of protein immunoreactivity. The total intensity of immunoreactivity was summed for each serum screened and subsequently plotted in bar chart format, shown in Figure 16. The bar chart is grouped by vaccination route, and date sera were drawn post-LVS vaccination, with a horizontal bar indicating the mean intensity value for a particular group. From this graph, it is apparent that the total intensity of protein immunoreactivity varies between animals and the mean values vary between the vaccination groups. The mean intensity of protein immunoreactivity values are higher for the SC LVS-vaccinated animals, compared with the mean for groups of animals LVS-vaccinated by scarification. Within the SC-vaccinated groups of animals, a small, but not statistically significant increase in immunoreactivity in for SC vaccinated animals between 42 and 63 days post vaccination was observed. By comparison, the mean observed intensity of immunoreactivity for rabbits LVS-vaccinated by scarification did not differ greatly between sera drawn at 42 or 63 days post-vaccination. Animals that survived challenge with SCHU S4 are indicated by asterisk (*) in Figures 15 and 16. No clear pattern was evident from the simple analysis of these data; surviving animals did not appear to generate antibodies towards specific proteins, nor a greater intensity of reactivity than those that succumbed to challenge (Figure 15 and 16).

From the human immunoproteomics work described in Example 3, eleven proteins were downselected as commonly reactive antigens, reactive with both human patient and vaccinee sera, with a minimum frequency of 30 %. These include dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077), 50S ribosomal protein L7/L12 (FTT_0143), 30S ribosomal protein S1 (FTT_0183), DNA-directed RNA polymerase alpha subunit (FTT_0350), Acetyl-CoA carboxylase (FTT_0472), Outer membrane associated protein FopA (FTT_0583), Peroxidase/catalase FTT_0721c), Chaperone protein DnaK (FTT_1269c), Pyruvate dehydrogenase E2 component (FTT_1484c), Chaperone protein groEL (FTT1696), Hypothetical membrane protein (FTT_1778c). When comparing the

repertoire of immunoreactive proteins identified from 2D Western blots probed with sera from LVS-vaccinated rabbits and non-human primates, with those identified from human patients and LVS vaccinees, the same eleven proteins were observed to be reactive with sera from all species. Table 6 lists these proteins and the frequency with which they were observed to be reactive with sera from each animal species. For the NHP immunoproteomics, this was subdivided into the frequency of reactivity with those animals surviving challenge and those that succumbed to challenge with SCHU S4.

Table 6. *Francisella* proteins found to be reactive with sera from LVS-vaccinated humans and animal models, and infected humans. The locus tag refers to the SCHU S4 genome sequence; for each group the number of subjects and the number of sera in which reactivity towards a specific protein was observed. For the NHP study, the frequency of reactivity with animals surviving challenge, and those succumbing to SCHU S4 challenge is indicated.

Locus tag	Protein Name	Vaccine NDBR lot 11 LVS (n=8)	Vaccine DVC lot 17 LVS (n=8)	Type B convalescent (n=12)	Type A convalescent (n=12)	NHP (n=40)*	Rabbit (n=40)
FTT_0077	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	8	7	11	8	26, 7	27
FTT_0143	50S ribosomal protein L7/L12	8	6	8	10	19, 2	38
FTT_0183	30S ribosomal protein S1	7	5	7	-	26, 7	27
FTT_0350	DNA-directed RNA polymerase alpha subunit	5	3	4	-	29,10	40
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	6	3	8	7	23,1	15
FTT_0721c	Peroxidase/catalase	5	5	6	7	13,7	27
FTT_1269c	Chaperone protein dnaK	5	7	5	8	24,7	21
FTT_1484c	Pyruvate dehydrogenase E2 component	4	3	3	10	25, 6	16
FTT_1696c	Chaperone protein, groEL	4	6	12	9	20, 4	31
FTT_1778c	Hypothetical membrane protein FTT_1778c	8	6	8	5	19, 2	38
FTT_0583	Outer membrane associated protein fopA	5	3	4	-	29, 10	

* 30 survivors, 10 non-survivors of SCHU S4 challenge

Example 6: Immunoproteomics in murine model of tularemia

The murine model of tularemia used in this Example has been described in the literature, and is the most widely used tularemia model. However, concerns regarding how applicable findings in mice are to humans exist, not least because LVS remains virulent for mice. For example, *F. tularensis* LVS ATCC 29684 inoculated intradermally (ID) elicits a similar sub-lethal infection in the skin, liver, and spleen of both BALB/c and C57BL/6 mice that persists for approximately 2 weeks. LVS also differs in the ability to protect mice from challenge with type A strains of the pathogen, depending upon the genetic background of the mouse. LVS infection renders BALB/c mice immune to a subsequent systemic challenge with >100 LD_{50} of a virulent type A strain of *F. tularensis*, it fails to protect C57BL/6 mice from a 100-fold smaller challenge.

Earlier work compared the immunoproteomes of the two LVS-vaccinated mouse strains aimed to determine whether successful immunization of BALB/c mice with lot 16 LVS results in seroconversion to a unique subset of immunoreactive proteins (Twine et al., 2010). Twenty-eight proteins were observed to be immunoreactive with sera from successfully and unsuccessfully vaccinated BALB/c mice, including the following proteins observed to be reactive with sera from other animal modes of tularaemia: Outer membrane associated protein FopA (FTT0583), Chaperonin GroEL (FTT1696), Dihydrolipoamide succunyl transferase component of 2-oxoglutarate dehydrogenase complex (FTT0077), and acetylCoA carboxylase (FTT_0472).

Intranasal and subcutaneous vaccination of BALB/c mice with LVS

Development of the murine model of tularemia at the National Research Council Canada included a direct comparison of routes of LVS vaccination (Lot 17 LVS), including subcutaneous (SC) and intranasal (IN) of BALB/c mice. For each vaccination route, a cage of mice (n=5) were vaccinated (IN dose was $1x10^3$ cfu; SC dose was $1x10^4$ cfu) and sera drawn four weeks post-vaccination. Immunoreactive proteins were detected and identified as described in Examples 2 and 3.

The results (Figure 17) show that selected marker proteins of the present invention are reactive with post-vaccination sera, regardless of the route of vaccination. Specifically, the immunoreactive proteins identified are listed below, based on the route of vaccination.

Intranasal vaccination: the proteins dihydrolipoamide succinyltransferase (FTT_0077), 30S ribsosomal protein S1 (FTT_0183), GroEL (FTT_1696), DnaK (FTT_1296c), Acetyl CoA carboxylase (FTT_0472) were reactive with sera from all vaccinated mice screened. The

protein Catalase (FTT_721c) was reactive with all but one of the sera screened. In addition, the outer membrane protein FopA (FTT_0583) was reactive with some of the sera screened.

Subcutaneous vaccination: the proteins GroEL (FTT_1696) and DnaK (FTT_1269c) were observed to be reactive with all sera screened. In addition, the proteins dihydrolipoamide succinyltransferase (FTT_0077) and 30S ribsosomal protein S1 (FTT_0183) were reactive with the majority of sera drawn.

Comparison of the profile of immunoreactive proteins with antisera generated by subcutaneous vaccination of BALB/c mice with LVS and an LVS Δ*IglC* mutant.

To compare markers from a protected and an unprotected mouse, BALB/c mice that can be successfully vaccinated with LVS were also vaccinated with a mutant of LVS (LVS Δ*IglC*) that is further attenuated and does not protect BALB/c mice from intradermal challenge with virulent SCHU S4. Comparison of the repertoire of immunoreactive proteins from successfully and unsuccessfully vaccinated mice has the potential to reveal antigenic proteins that are markers of protective vaccination. Vaccination was performed as described above (SC vaccination, dosage for LVS and mutant strains was $1x10^4$ cfu), and detection and identification of immunoreactive proteins was performed as described in Examples 2 and 3.

The immunoreactive proteins observed when Western blots were aligned with protein stained 2D-PAGE were very similar to those observed in the other murine immunoproteomics studies described herein. Figure 18 shows representative blots probed with sera form unvaccinated control mice (Figure 18A), mice vaccinated SC with LVS (Figure 18B) and mice vaccinated SC with LVS Δ*IglC* (Figure 18C). Notable differences between the two sets of blots were observed, especially when comparing the observed intensity of individual immunoreactive areas. For example in most murine sera screened, areas of immunoreactivity corresponding to dihydrolipoamide succinyltransferase and 30S ribosomal protein S1 showed much greater intensity of immunoreactivity in LVS-vaccinated mice, compared with LVS Δ*IglC*-vaccinated mice. In addition, areas of immunoreactivity corresponding to peroxidase/catalase and GroEL were also observed to have greater intensities of reactivity with sera from LVS-vaccinated mice. Of particular note, immunoreactivity towards the protein DnaK was only observed at detectable levels in sera from LVS-vaccinated mice.

Sequences

SEQ ID NO: 1 – FTT_0077 - dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (Francisella tularensis subsp. tularensis SCHU S4)

MVELKVPMFPESVADGTLAQWNKNEGDFVNEGDILAEIETDKVVLEVPATSSGVLKGIKKHAG
DTVLSEESLAIIDTAVSTSEPNQQTTNQGNASEATATGQEIDIKAPVFPESVADGTISEWHKKE
GEAVSEGDILAEIETDKVVLEVPATSNGVLTKILKTAGETVLSAELIAKITAGGATATTKSEASVG
VSQANNDPHLVPSARKAFNASGLDTAANIEGTGKKGRITSEDVKKAVASVNKPQQQTVVINQ
GARYEKRVKMTRLRQTIANRLVEVQHTNAILTTFNEVDMSAVMELRNKYKDMFVKEHDTKLG
FMSFFIKAATEALKKFPDVNASIDGDEIVYHNYFDIGIAVGTDRGLVVPVLRDTDTKSLAELEAD
VLDKAIKGRDGKLSLEDMQGGTFTITNGGTYGSMLSTPIINSPQSAILGMHNIVERPVVVKGEIK
IRPIMYLALSYDHRIIDGGTSVRFLKMIKELIEDPNRILLQV

SEQ ID NO: 2 – FTT_0143 - 50S ribosomal protein L7/L12 (Francisella tularensis subsp. tularensis SCHU S4)

MAITKEDILNAVAEMSVMDVCDLVKMMEDKFGVSAAAAVAVAAGPVAGPAEAAEEKTEFDVV
LVDAGSNKIAAIKAVRGATGLGLKEAKDAVEGTPFTVKEAASKEEAEVLKKQLEEAGAKVELK

SEQ ID NO: 3 – FTT_0183 - 30S ribosomal protein S1 (Francisella tularensis subsp. tularensis SCHU S4)

MSENFKELFEQSLKQTEMRIGKIIEATVVSIDKEFAMIDAGLKSESFIPVSSLKNSNGELEVAAG
DRINVVLEALDNSCGETRLSRDKAKKIELWDRIEKAFENNETVLGKITNHVRGGYTMDVEGLR
AFLPGSLVDTRPIKDVAHLEDKDIELKVVKIDTKRNNIVVSRKAVIEENNSGDRDAMLEKISEGS
VLKGIVKNITDFGAFIDLGGVDGLLHITDISWSRISHPTDVLSIGQEIDVKVIKFDKEKQRISLGIK
QLGEDPWLNIANELPVGAKLMGTVTNITDYGCFVKLKEGIEGLVHTSEMDWTNKNVNPHKAV
SIGQEIEVIVLELDADNHRISLGIKQCRPNPWSEFEKNYKPGDKVTGKIRSITEFGVFIGLEGGID
GLVHISDVAWDNPAKAIKELKKGDEVEAVLVSVNTDLERIALSMKQLSEDPFKNFINIHPKGSL
VTGKVTKVQDNGAVVMLDEDNNIDGFIRISEISAEHTKDVRDELSEGQEVEARIINIDAKKRSITL
SIKAVDEDNTAAGKSNYKVEQMTPTTLGDLIKEQLNKK

SEQ ID NO: 4 – FTT_0350 - DNA-directed RNA polymerase alpha subunit (Francisella tularensis subsp. tularensis SCHU S4)

MSNNNSKLEFVPNIQLKEDLGAFSYKVQLSPVEKGMAHILGNSIRRVLLSSLSGASIIKVNIANV
LHEYSTLEDVKEDVVEIVSNLKKVAIKLDTGIDRLDLELSVNKSGVVSAGDFKTTQGVEIINKDQ
PIATLTNQRAFSLTATVSVGRNVGILSAIPTELERVGDIAVDADFNPIKRVAFEVFDNGDSETLE
VFVKTNGTIEPLAAVTKALEYFCEQISVFVSLRVPSNGKTGDVLIDSNIDPILLKPIDDLELTVRS
SNCLRAENIKYLGDLVQYSESQLMKIPNLGKKSLNEIKQILIDNNLSLGVQIDNFRELVEGK

SEQ ID NO: 5 – FTT_0472 - Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (Francisella tularensis subsp. tularensis SCHU S4)

MDLLKAIDRVAEILNSSDIKEIRIKDGGSSIFMTKNNTAAITSVVFAAPVASNVASAAPAVATAAT
SAAAPKVNLAEEISGEEIKSPMVGTFYGASSPDAAPYVKEGQEVKKGDVLCIIEAMKIMNKIEA
ERAGKIVKIIAKDGEPVQFDQPLFIIE

SEQ ID NO: 6 – FTT_0583 - Outer membrane associated protein, fopA (Francisella tularensis subsp. tularensis SCHU S4)

MMRLKSIVIATTVLLGSATASIAAGSDNIDTLANTNSATTQSSGFAANNFIAPFANTYSALTNKD
NTWGPQDRTGQWYLGVDANGLAGTPNSPSGAGANFTIGYNINKYFAVQYNQLVGRVFAGLG
EGVVNFSNNTMFTPYAAGGAGWANLAGQATGAWDVGGGLKFELSRNVQASVDYRYIQTMA
PSNISGANGRAGTNMIGAGLTWFFGGKDTTNNDTGNIQDNGATTAAQTVAMPTIDESKYVLP
AGIKQCEGNFNLTEDGVACYTINGDDVTVYLDTKFAYDKATLNAKGKKAIASFVNFIKDSNISSV
TVKGYASQGQTGSEFDIYNQKLSEKRAQAVADYMKQLGLDSEKIITKGFGYNDTLGGIHKSDP
RNQRVEASVSAPLKEAN

SEQ ID NO: 7 – FTT_0721c - Peroxidase/catalase (Francisella tularensis subsp. tularensis SCHU S4)

MLKKIVTALGMSGMLLASSNAIAEDTTTKNDNLSPQSVDLSPLRNLNKLDSPMDKDYNYHQAF
KKLDTEQLKKDMQDLLTQSQDWWPADFGNYGPFFIRLSWHDAGTYRIYDGRGGANRGQQR
FSPLNSWPDNVNLDKARQLLWPIKQKYGDAVSWSDLIVLAGTVSLESMGMKPIGFAFGREDD
WQGDDTNWGLSPEEIMSSNVRDGKLAPAYAATQMGLIYVNPEGPDGKPDIKGAASEIRQAFR
AMGMTDKETVALIAGGHTFGKTHGAVPEDKVKQAIGPAPDKAPIEQQGLGWHNSYGTGNGD
DTMGSGLEGSWTSTPTFWNHDFLHNLYNLDWKKTLSPAGAHQWTPTNAKPENMVPDAHKP
GVKHKPIMFTTDLALKEDDGFNKYTQEFYNNPEEFKEEFAKAWFKLTHRDMGPKSRYIGPWIP
EQNFIWQDPVPAADYKQVSTQDIAQLEQDIINSGLTNQQLIKTAWDSASTYRKTDYRGGSNGA
RIALAPEKDWQMNEPAKLEVVLTKLKEIQTNFNNSKTDGTKVSLADLIVLGGNVGVEQAAKQA
GYNIQMPFVPGRTDATQAQTDIESFNYLKTKSDGFINYTDGSVSADKLPQTLVEKASMLDLNIP
EMTVLVGGMRALDVNYDNSQEGVLTTTPGQLNNSFFVNLLDMSTQWKKSDKKDGEYIGIDRK
TGKQKWTASPVDLIFGSNSELKAVAQVYAENGNEQKFVNDFAKAWHKVMMLGRFDVQQ

SEQ ID NO: 8 – FTT_1269c - Chaperone protein dnaK (Francisella tularensis subsp. tularensis SCHU S4)

MGKIIGIDLGTTNSCLAIMDGKTAKVIENAEGHRTTPSVVAYTDSGEILVGQAAKRQAVTNPDNTFFAIK
RLIGRKYDDKAVQEDIKKKVPYAVIKADNGDAWVATKEGKKMAPPQVSAEVLRKMKKTAEDYLGEPVTE
AVITVPAYFNDSQRQATKDAGKIAGLEVKRIINEPTAAALAYGVDSKKGEQTVAVYDLGGGTFDISIIEIA
DVDGDNQIEVLSTNGDTFLGGEDFDLALMNYLIDEFKKEQGIDLHNDKLALQRVREAAEKAKVELSSAQ
QTDVNLPYITADATGPKHLNIKVTRAKFESLVSDLVMRSLEPCKKALEDAGLSKSDITEVLLVGGQTRMP
LVQEKVKEFFGKEPRKDVNPDEAVAVGAAIQGGVLAGDVKDILLLDVTPLSLGIETMGGVMTKLIERNTTI
PTKKSQVFSTAEDNQPAVTIHVLQGEREMASANKSLGRFDLADIPPAPRGMPQIEVTFDIDANGILNVSA
KDKATGKEQNIVIKSSSGLSEEDIEKMVQDAEANAEADKKFHDLVTARNTADNLIHSSRKAIQELGDKVT
AAEKEKIEEACKELEAATKGDDKQAIESKTKALEEAFAPIAQKAYAEQAQAAVAQGGAKAEEPKKEEDV
VDADFEDVEDDKK

SEQ ID NO: 9 – FTT_1484c - Pyruvate dehydrogenase E2 component (Francisella tularensis subsp. tularensis SCHU S4)

MSIEIVKVPDIGDYDNVDVIEVNVAVGDVIAEEDSLITLETDKASMEVPSPFAGKITKLTVKVGD
KVSQGTAIMEVEVESAADQAATTQSQPQTTSSAPVAATTNLIVDVEVPDIGDYDSVDVIEVSVK
VGDEIAEEDSLITLETYKASMEVPSPVAGKVVEVITKVGDKVSQGSLILKVETGSSAQAPAQEQ
SQQSAPVKSAAEEIIDVKVPDIGDYDSVDVIEVSVAVGDKIEEEDSLITLETDKASMEVPSPVAG
EVVEIITKVGDKVSQGSLILKVKTQGSAPVEQTSSQPAPAKQEQAKQQAATPAAPTPASSSVN
EYAVDNSNAHASPAVRKLARILNIDLSKVKATGRKGRVTKEDCYNYIKHAVTQVQTGKVAASG
SGLDLLDDPVVDFAKFGEIETQPLSRINKISAKNLHRNWVKIPHVTFYDDADVTDLEEFRNAKK
AFAEKKGIKITPLSFLVKAAAVALQEFPRFNSSLSNDGENLIIKKYYNIGFAADTPAGLMVPVVK
DADKKGIIEISKDIMELAGKARDGKLGAKDMTGATFTISSLGVLGTTSFTPIINMPEVAIMGVSKT
AVKPIWNGKEFIPRTMLPLSLSTDHRVIDGALAAKFLTRYCQILSDLREIIM

SEQ ID NO: 10 – FTT_1696c - Chaperone protein, groEL (Francisella tularensis subsp. tularensis SCHU S4)

MAAKQVLFSDEARAKMLDGVNTLANAVKVTLGPKGRNVVLDKSFGTPTITKDGVSVAKEIELE
DKFENMGAQIVKEVASKTADVAGDGTTTATVLAQALLTEGLKAVAAGMNPMDLKRGIDKATA
RLVEELKALSKPCSDPKSIEQVGTISANSDATVGKLIADAMAKVGKEGVITVEEGKGFEDELDV
VEGMQFDRGYLSPYFATNQENMTTDLENPYILIVDKKISNIRDLLPILEGVSKSGRALLIIAEDVE
SEALATLVVNNMRGVVKVCAVKAPGFGDRRKAMLEDIATLTGATFVSEDLSMKLEETNMEHL
GTASRVQVTKDNTTIIDGAGEKEAIAKRINVIKANIAEANSDYDREKLQERLAKLSGGVAVIKVG
AVTEAEMKEKKDRVDDALHATRAAVEEGIVAGGGVALIRAQKALDGLTGENDDQNYGIALLRK
AIEAPLRQIVSNAGGESSVVVNQVKANQGNYGYNAANDTYGDMVEMGILDPTKVTRSALQHA
ASIAGLMITTEAMIGEIKEAAPAMPMGGGMGGMPGMM

SEQ ID NO: 11 – FTT_1778c - Hypothetical membrane protein (Francisella tularensis subsp. tularensis SCHU S4)

MKLRKVLIATLLGASALSLSSCWLLVGAAVGGGTAAYISGEYSMNMSGSVKDIYNATLKAVQS
NDDFVITKKSITSVDAVVDGSIKVDSTSFYVKIEKLTDNASKVTIKFGTFGDQAMSATLMDQIQK
NL

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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CLAIMS:

1. A set of biomarkers for tularaemia, said set of biomarkers comprising one or more proteins selected from the group consisting of: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 50S ribosomal protein L7/L12, 30S ribosomal protein S1, DNA-directed RNA polymerase alpha subunit, Acetyl-CoA carboxylase, Outer membrane associated protein, fopA, Peroxidase/catalase, Chaperone protein dnaK, Pyruvate dehydrogenase E2 component, Chaperone protein groEL, Hypothetical membrane protein.

2. A method of evaluating immunity against tularaemia in a subject, comprising:

a. contacting serum from the subject with the set of biomarkers of claim 1;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. determining the protection against tularaemia based on immunoreactivity to the biomarkers.

3. The method of claim 2 wherein immunoreactivity is evaluated using two-dimensional polyacrylamide gel electrophoresis Western blotting, Francisella proteome microarray, ELISA screening or a dot-blot technique.

4. The method of claim 2 wherein immunoreactivity is evaluated using two-dimensional polyacrylamide gel electrophoresis Western blotting.

5. The method of claim 2 wherein the subject is a rodent or a primate.

6. The method of claim 2 wherein the subject is a human.

7. A method of evaluating vaccine efficacy, comprising:

a. contacting serum from a vaccinated model of tularaemia with the biomarkers of claim 1;

b. evaluating immunoreactivity of the serum to the biomarkers;

c. correlating the immunoreactivity to immunoprotective status of the model; and

d. predicting vaccine efficacy in human based on the correlation of step c.

8. The method of claim 7 wherein the model of tularaemia is an animal model.

9. The method of claim 8 wherein the animal model is a mouse model, a rabbit model or a primate model.

10. The method of claim 7 wherein the model of tularaemia is a human model.

11. The method of any one of claims 7 to 10 wherein immunoreactivity is evaluated using two-dimensional polyacrylamide gel electrophoresis Western blotting, Francisella proteome microarray, ELISA screening or a dot-blot technique.

12. The method of claim 11 wherein immunoreactivity is evaluated using two-dimensional polyacrylamide gel electrophoresis Western blotting.

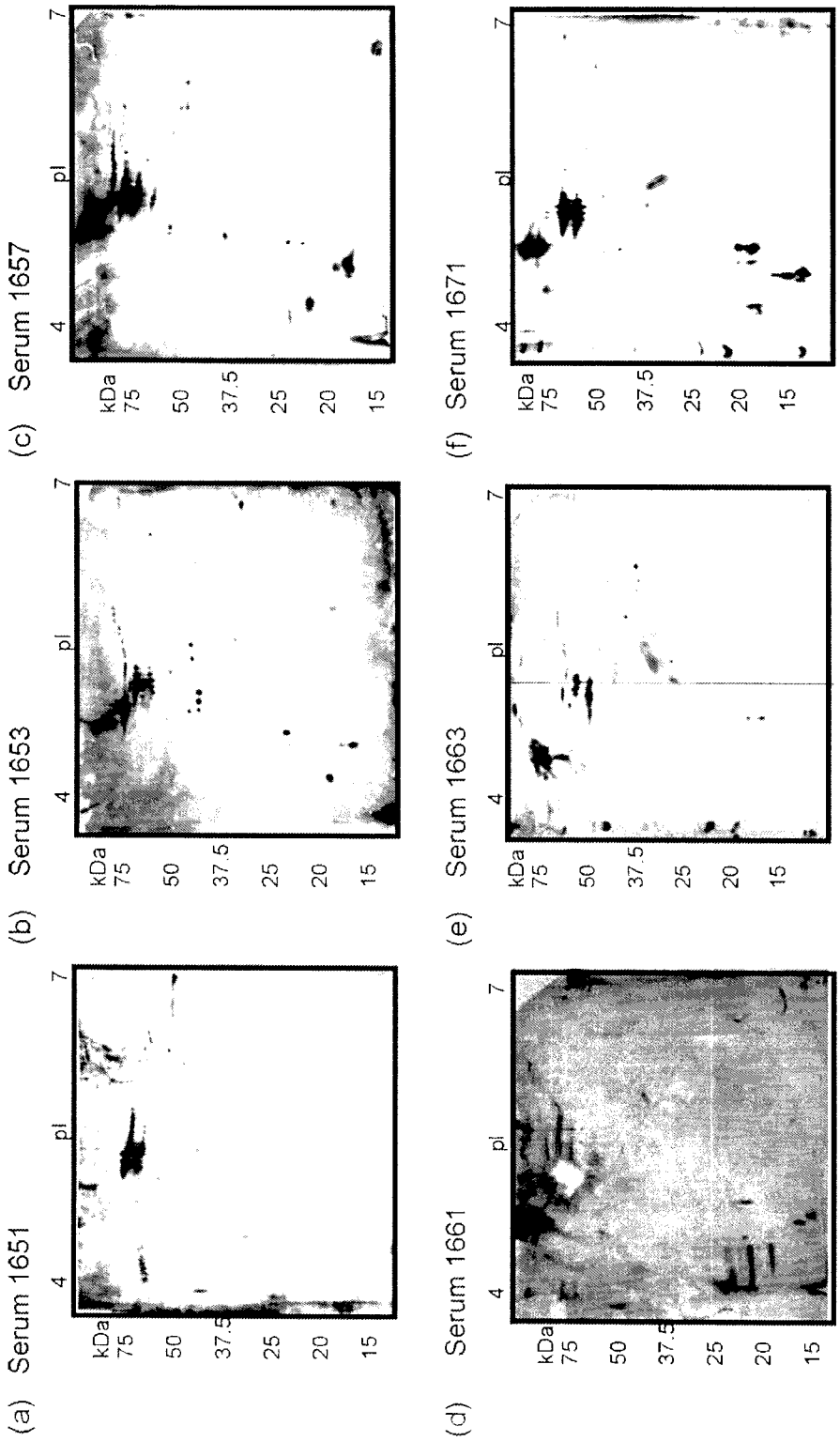

(a) Serum 1651
(b) Serum 1653
(c) Serum 1657
(d) Serum 1661
(e) Serum 1663
(f) Serum 1671
kDa
75
50
37.5
25
20
15
4
pI
7

FIG. 1

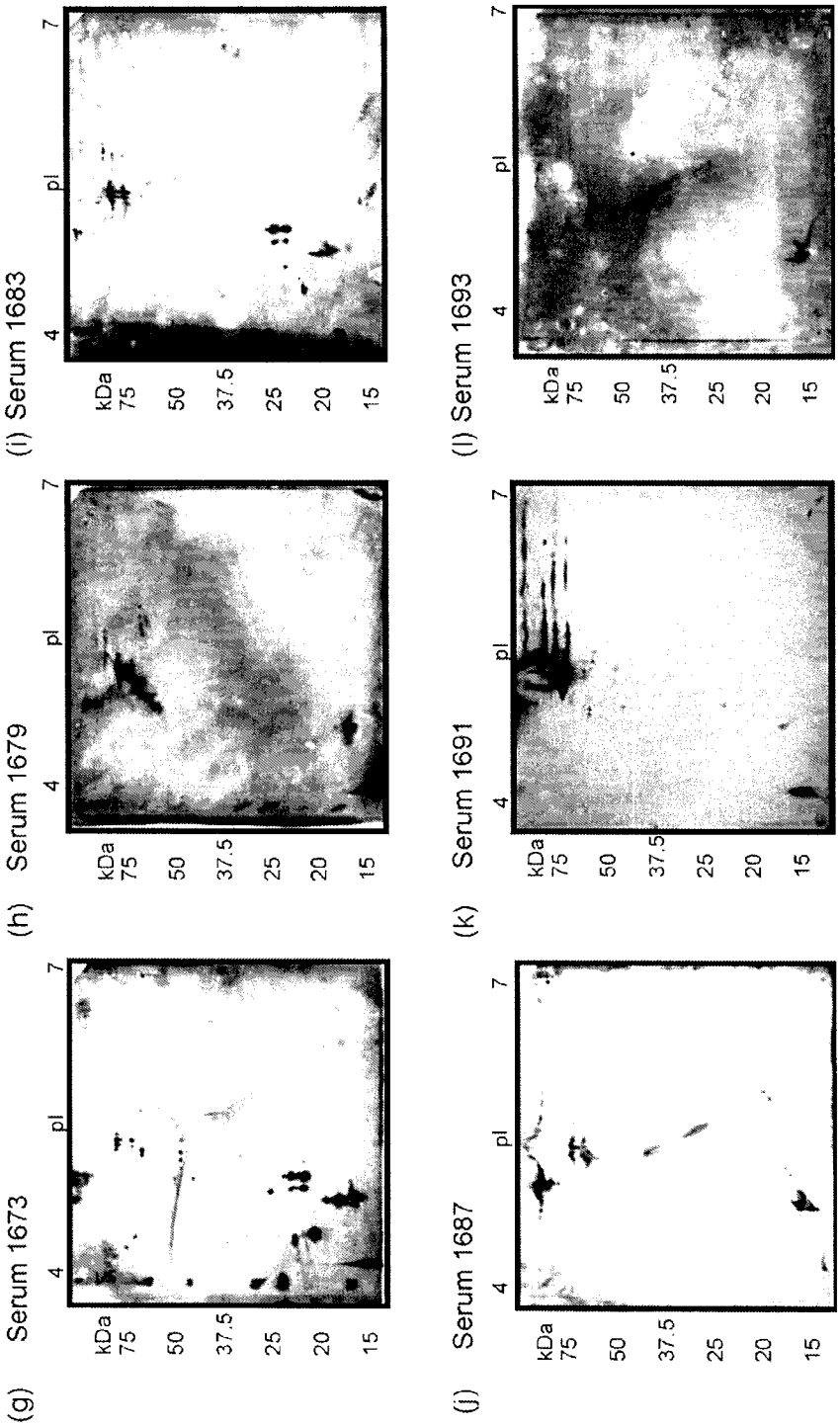

(g) Serum 1673
(h) Serum 1679
(i) Serum 1683
(j) Serum 1687
(k) Serum 1691
(l) Serum 1693
kDa
75
50
37.5
25
20
15
4
pI
7

FIG. 1

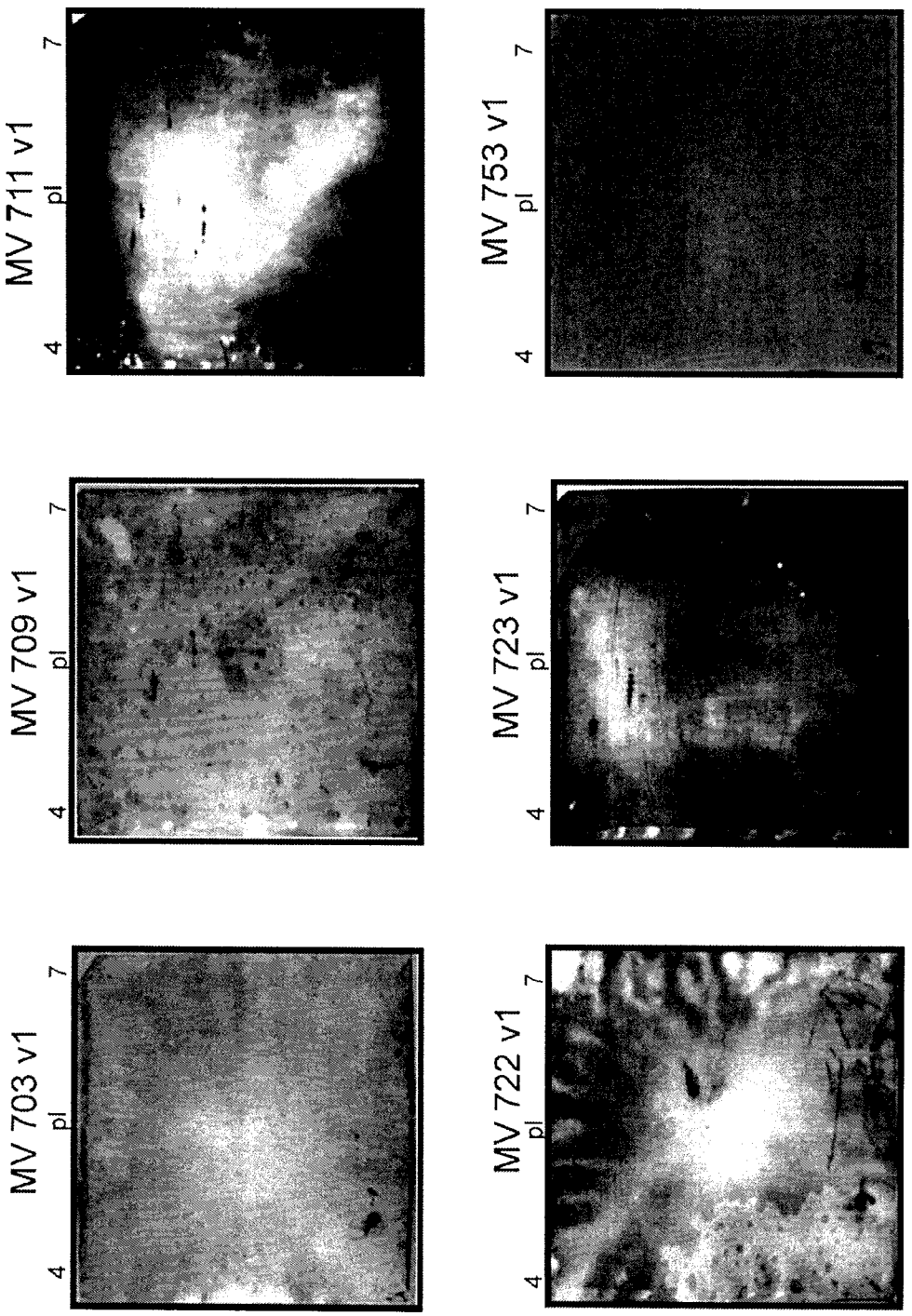

MV 711 v1
pI
4
7
MV 753 v1
pI
4
7
MV 709 v1
pI
4
7
MV 723 v1
pI
4
7
MV 703 v1
pI
4
7
MV 722 v1
pI
4
7

FIG. 2

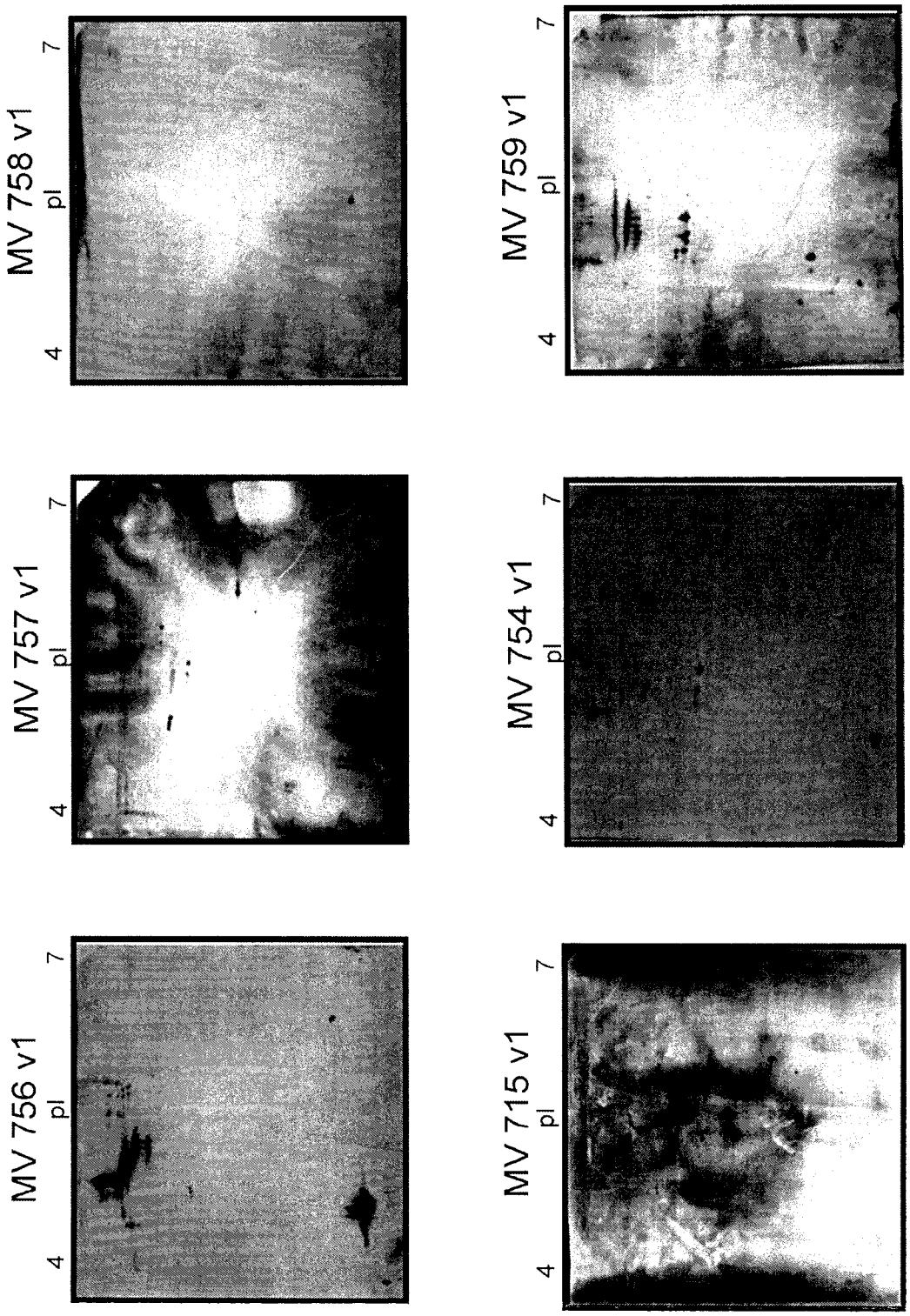

MV 758 v1
pI
4
7
MV 759 v1
pI
4
7
MV 757 v1
pI
4
7
MV 754 v1
pI
4
7
MV 756 v1
pI
4
7
MV 715 v1
pI
4
7

FIG. 2

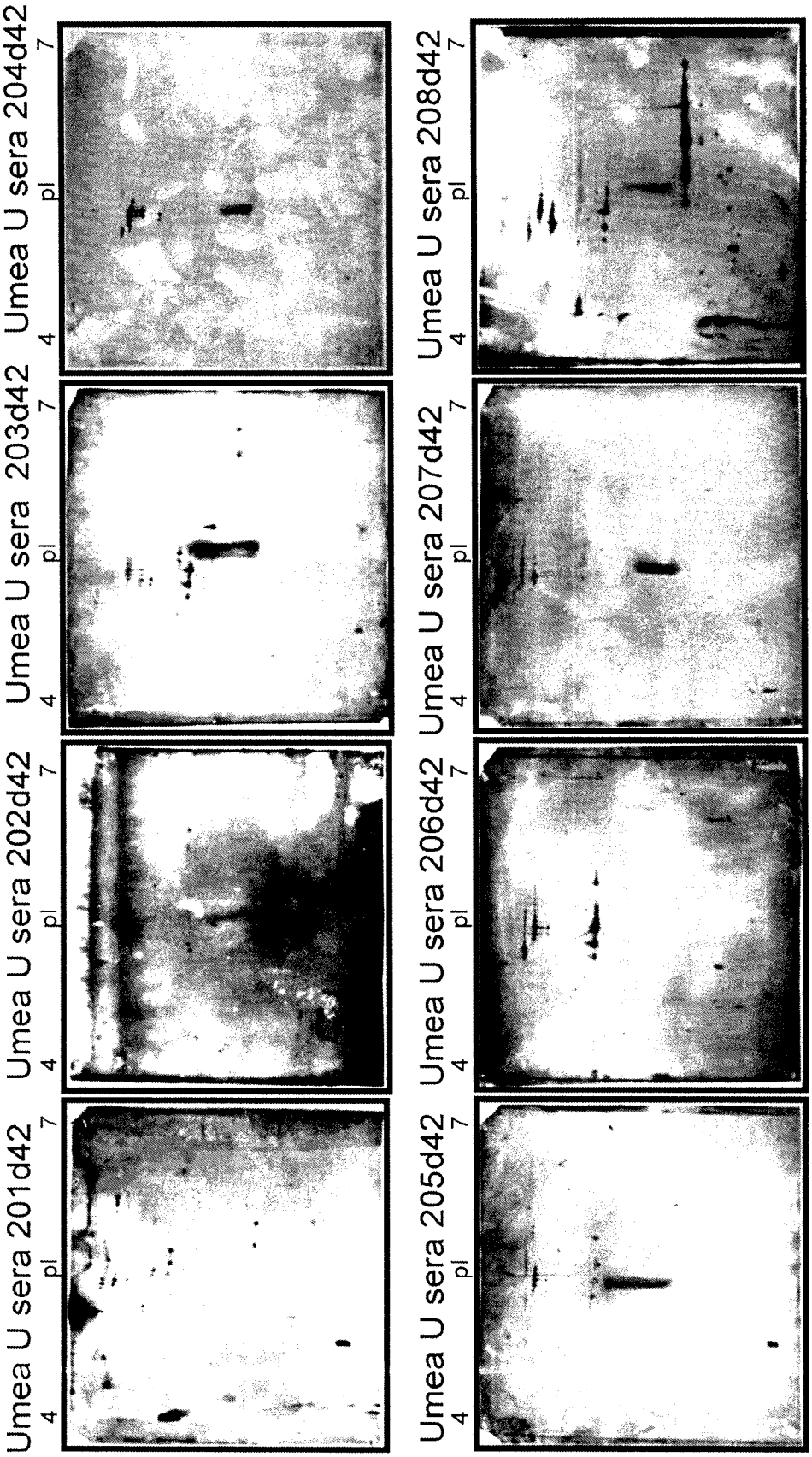

Umea U sera 201d42
Umea U sera 202d42
Umea U sera 203d42
Umea U sera 204d42
Umea U sera 205d42
Umea U sera 206d42
Umea U sera 207d42
Umea U sera 208d42
pI
4
7

FIG. 3

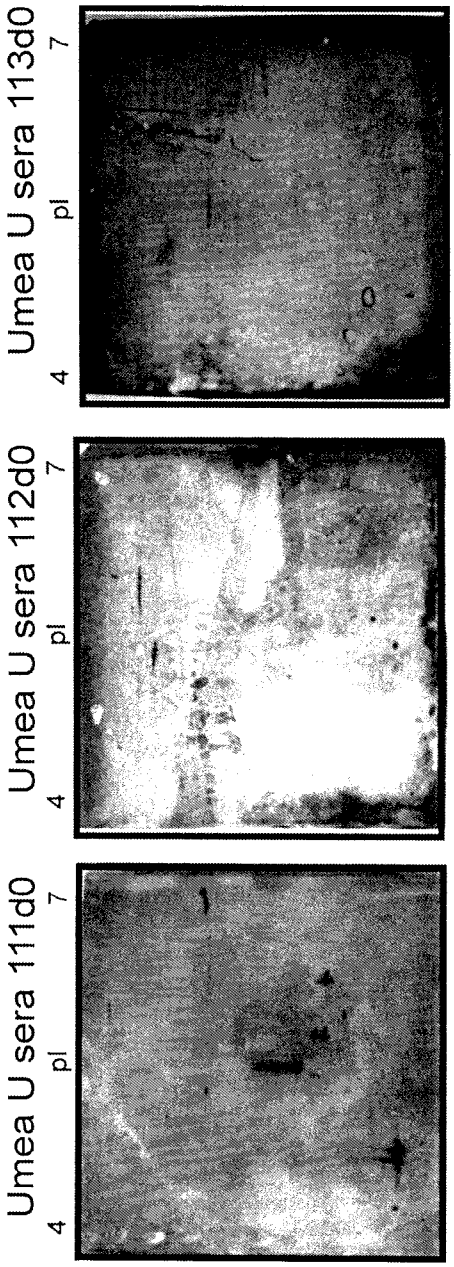

Umea U sera 111d0
4
pI
7
Umea U sera 112d0
4
pI
7
Umea U sera 113d0
4
pI
7
Umea U sera 114d0
4
pI
7
Umea U sera 115d0
4
pI
7

FIG. 3

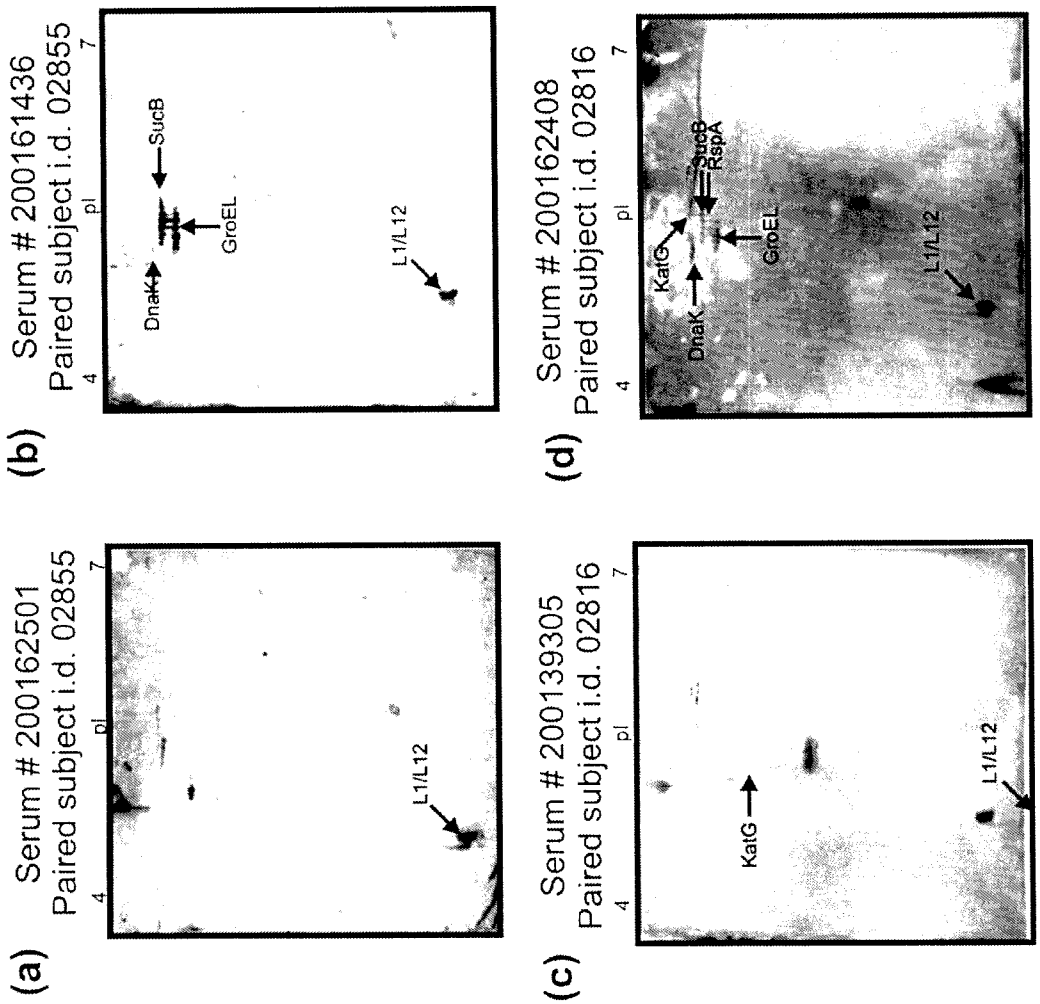

(a)
Serum # 200162501
Paired subject i.d. 02855
pI
4
7
L1/L12
(b)
Serum # 200161436
Paired subject i.d. 02855
pI
4
7
DnaK
SucB
GroEL
L1/L12
(c)
Serum # 200139305
Paired subject i.d. 02816
pI
4
7
KatG
L1/L12
(d)
Serum # 200162408
Paired subject i.d. 02816
pI
4
7
KatG
DnaK
SucB
RspA
GroEL
L1/L12

FIG. 4

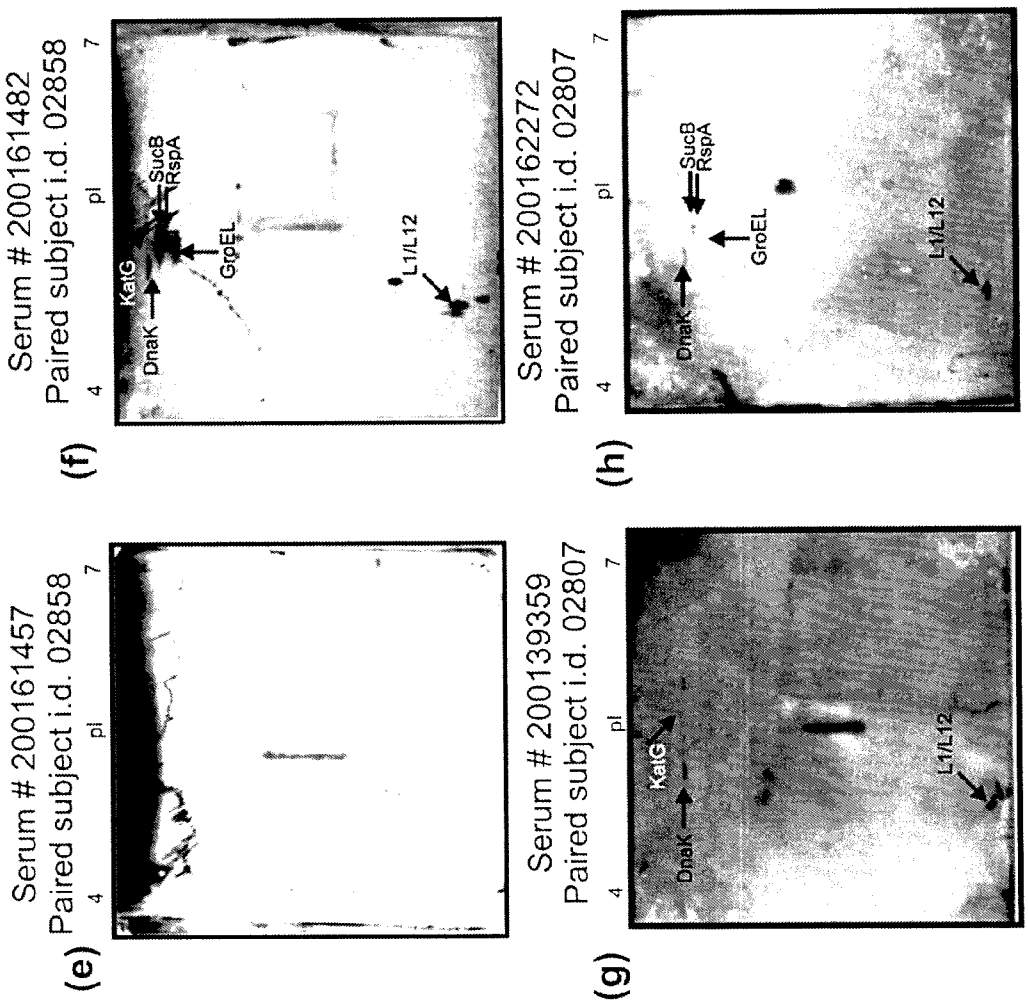

(e)
Serum # 200161457
Paired subject i.d. 02858
4
pI
7
(f)
Serum # 200161482
Paired subject i.d. 02858
KatG
DnaK
SucB
RspA
GroEL
L1/L12
(g)
Serum # 200139359
Paired subject i.d. 02807
(h)
Serum # 200162272
Paired subject i.d. 02807

FIG. 4

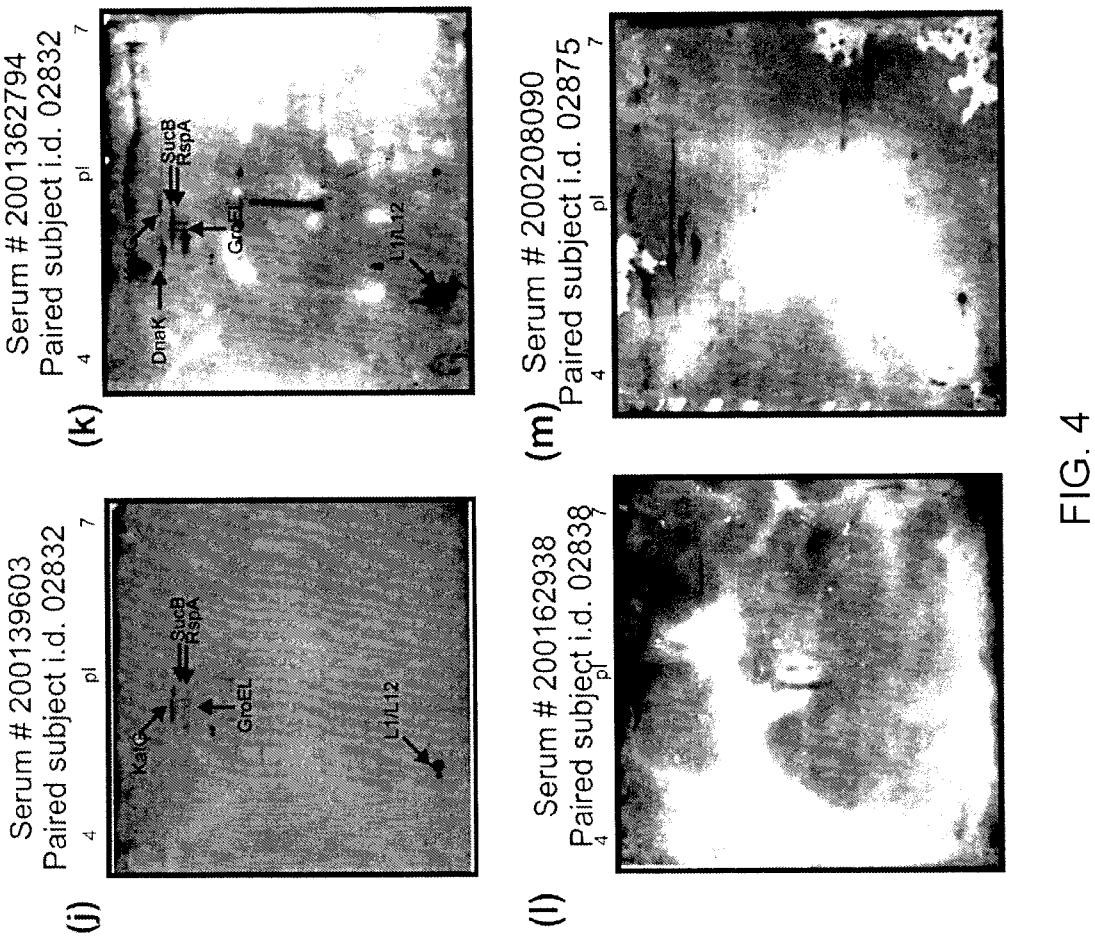

(j)
Serum # 200139603
Paired subject i.d. 02832
4
pI
7
KatG
SucB
RspA
GroEL
L1/L12
(k)
Serum # 2001362794
Paired subject i.d. 02832
4
pI
7
DnaK
SucB
RspA
GroEL
L1/L12
(m)
Serum # 200208090
Paired subject i.d. 02875
4
pI
7
(l)
Serum # 200162938
Paired subject i.d. 02838
4
pI
7

FIG. 4

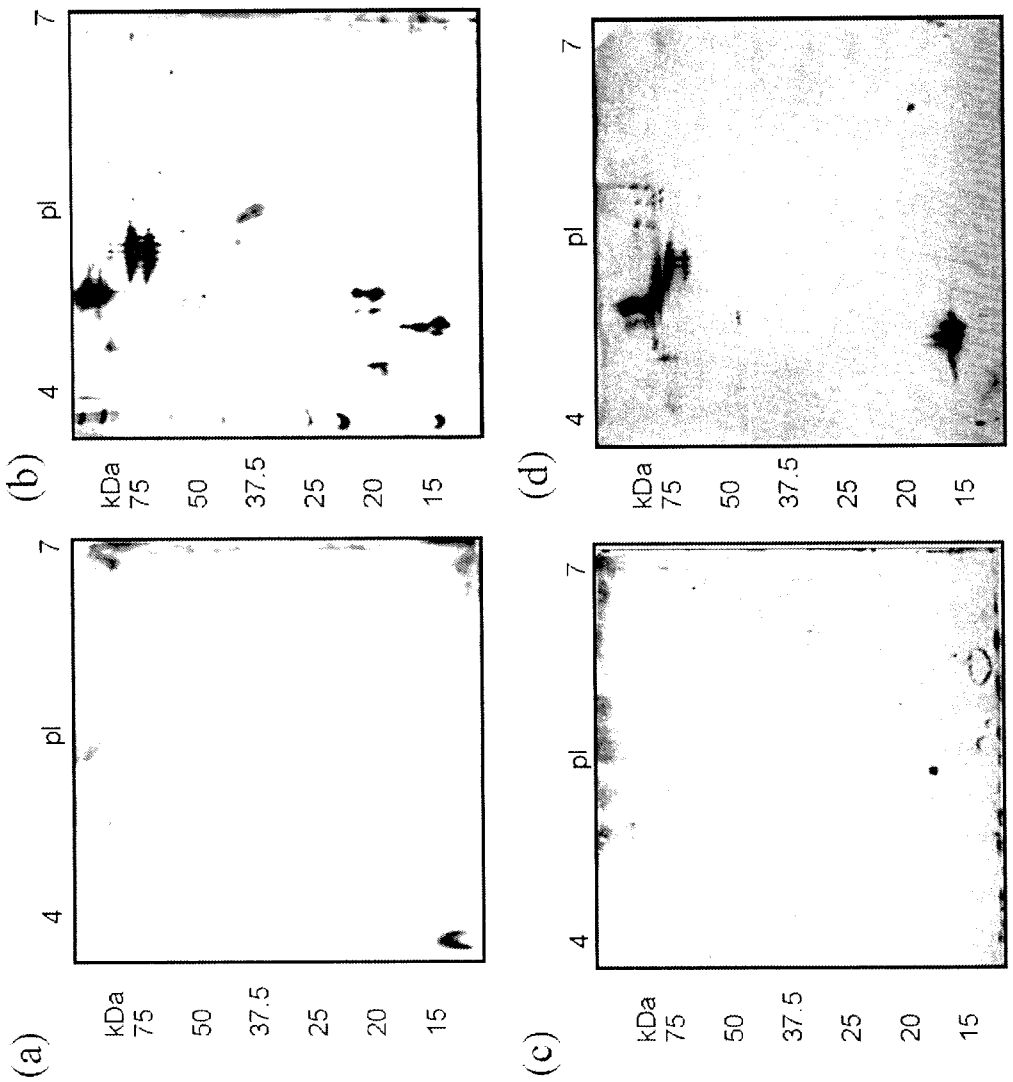


FIG. 5

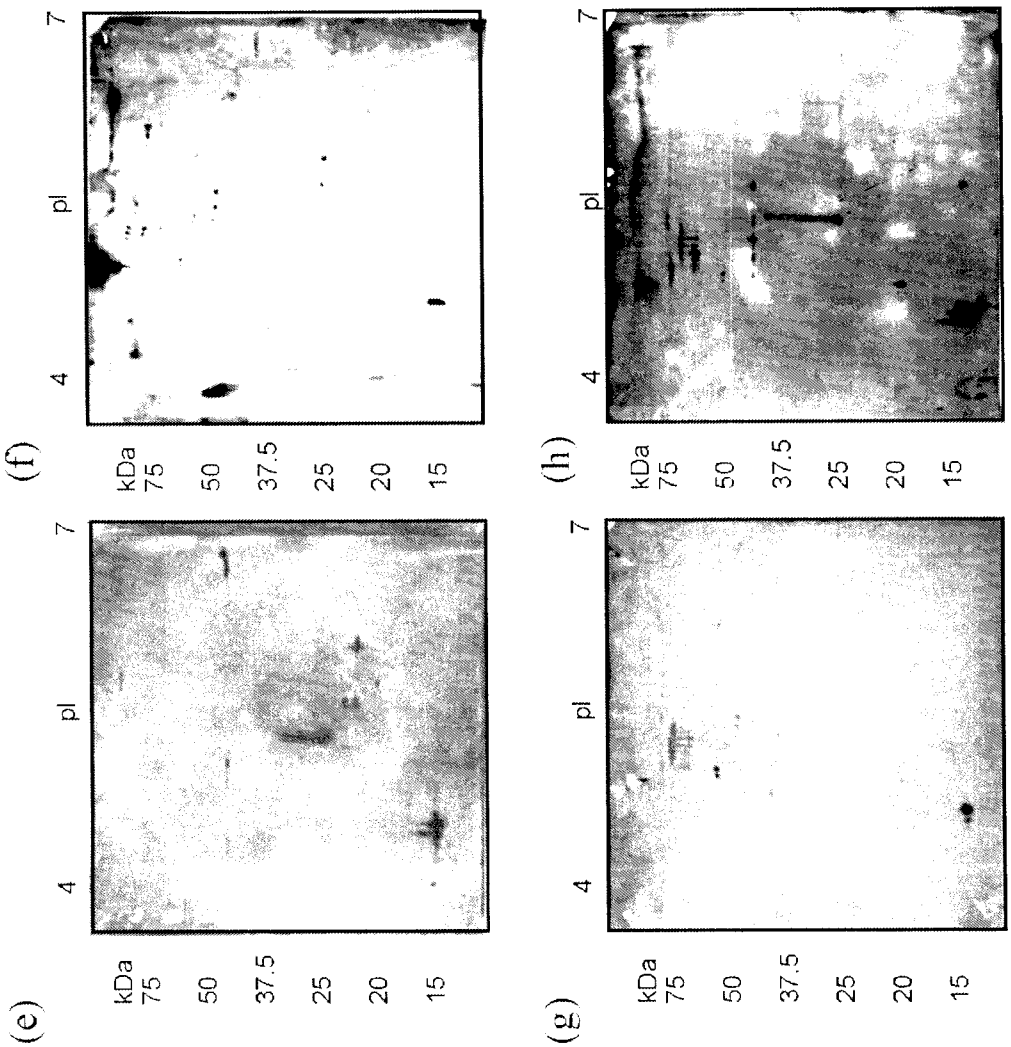


FIG. 5

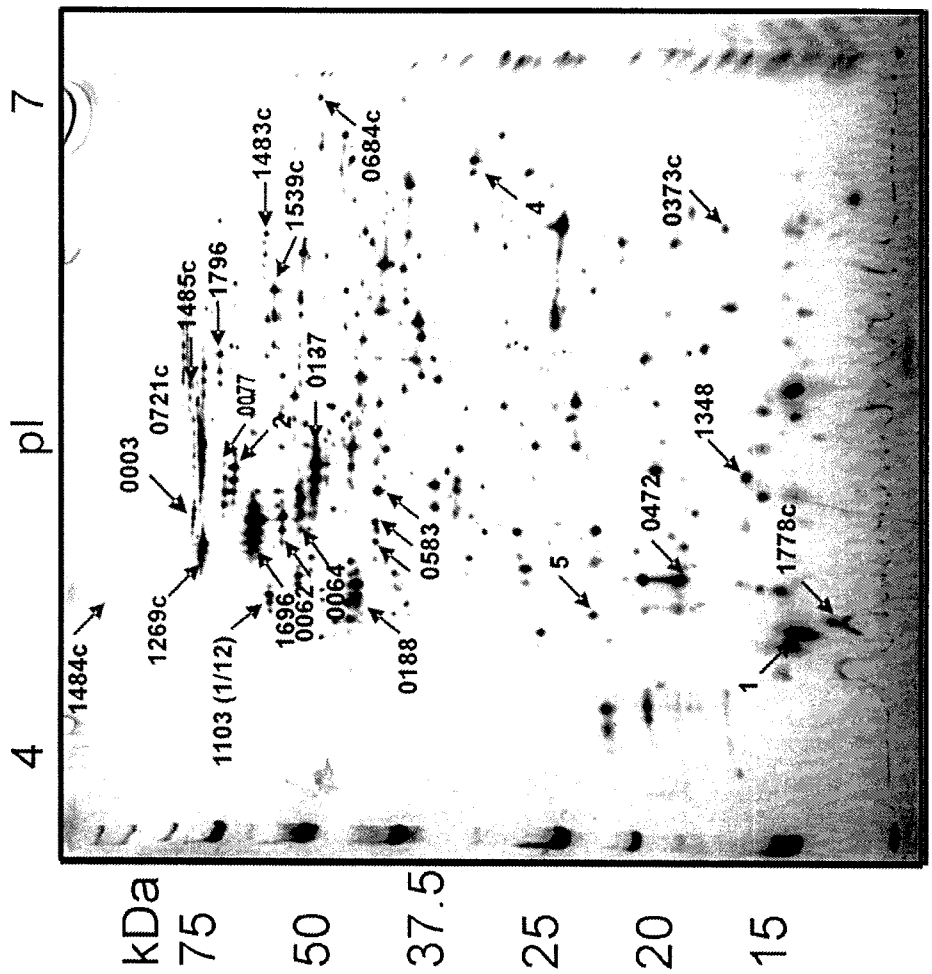

4
pI
7
kDa
75
50
37.5
25
20
15
1484c
1269c
1103 (1/12)
0003
0721c
1485c
1796
1696
0062
0064
0077
0137
0188
0583
1483c
1539c
0684c
0472
0373c
1348
1778c
1
2
4
5

FIG. 6

A

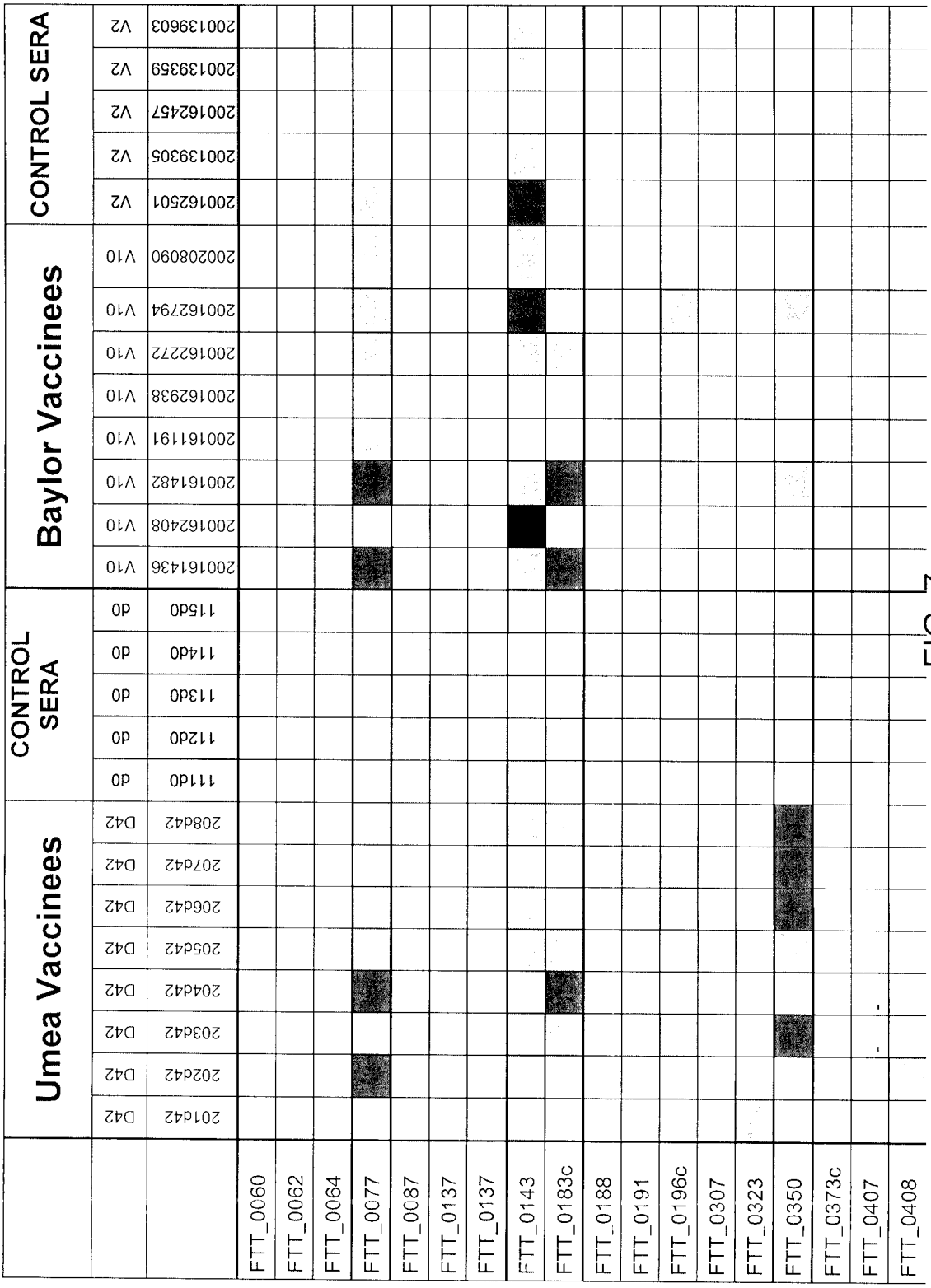

Umea Vaccinees
CONTROL SERA
Baylor Vaccinees
CONTROL SERA
201d42 D42
202d42 D42
203d42 D42
204d42 D42
205d42 D42
206d42 D42
207d42 D42
208d42 D42
111d0 d0
112d0 d0
113d0 d0
114d0 d0
115d0 d0
20016436 V10
20016208 V10
20016148 2 V10
20016119 1 V10
20016293 8 V10
20016227 2 V10
20016279 4 V10
20020809 0 V10
20016250 1 V2
20013930 5 V2
20016245 7 V2
20013935 9 V2
20013960 3 V2
FTT_0060
FTT_0062
FTT_0064
FTT_0077
FTT_0087
FTT_0137
FTT_0137
FTT_0143
FTT_0183c
FTT_0188
FTT_0191
FTT_0196c
FTT_0307
FTT_0323
FTT_0350
FTT_0373c
FTT_0407
FTT_0408

FIG. 7

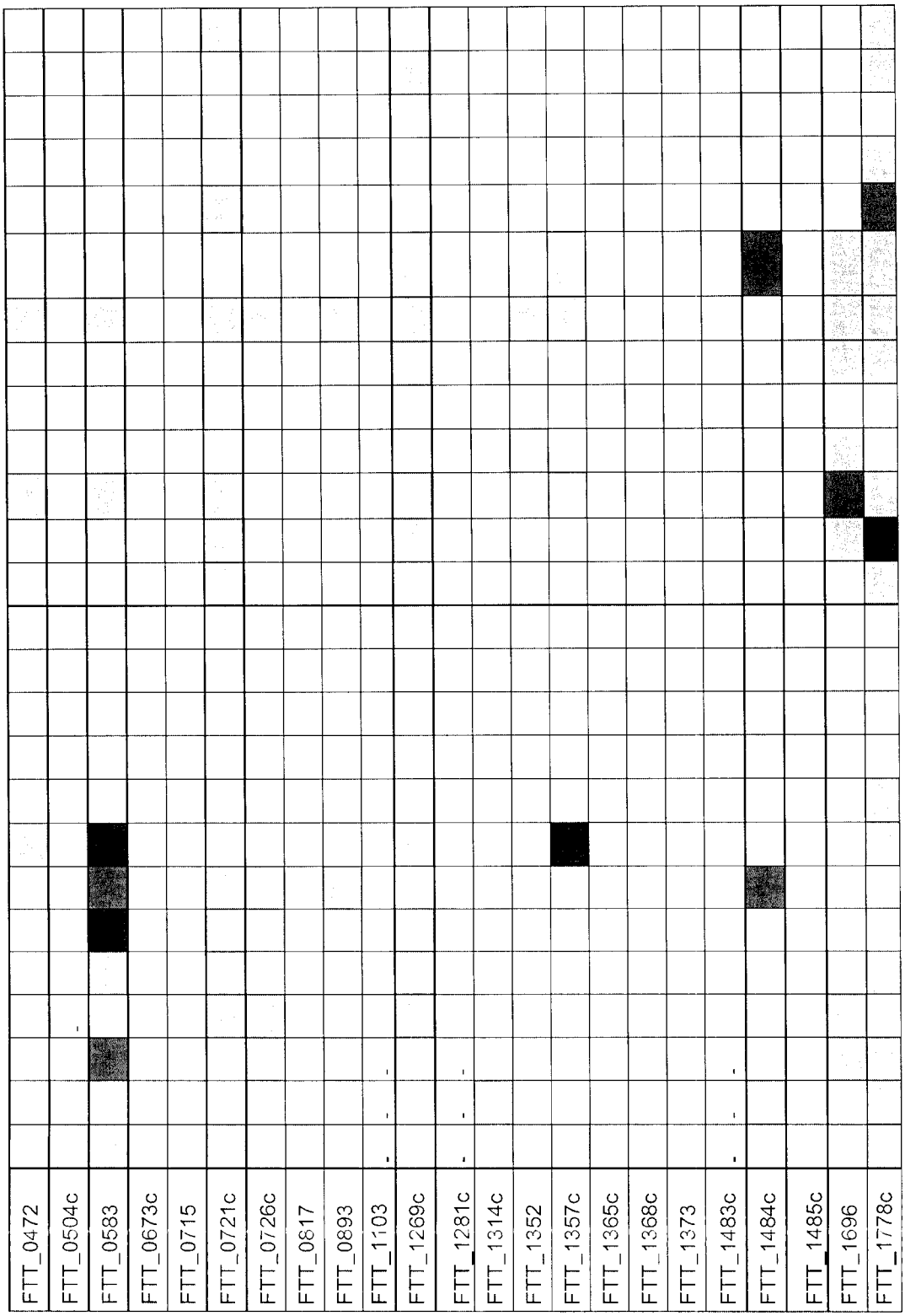

FTT_0472
FTT_0504c
FTT_0583
FTT_0673c
FTT_0715
FTT_0721c
FTT_0726c
FTT_0817
FTT_0893
FTT_1103
FTT_1269c
FTT_1281c
FTT_1314c
FTT_1352
FTT_1357c
FTT_1365c
FTT_1368c
FTT_1373
FTT_1483c
FTT_1484c
FTT_1485c
FTT_1696
FTT_1778c

FIG. 7 CONT'D

A

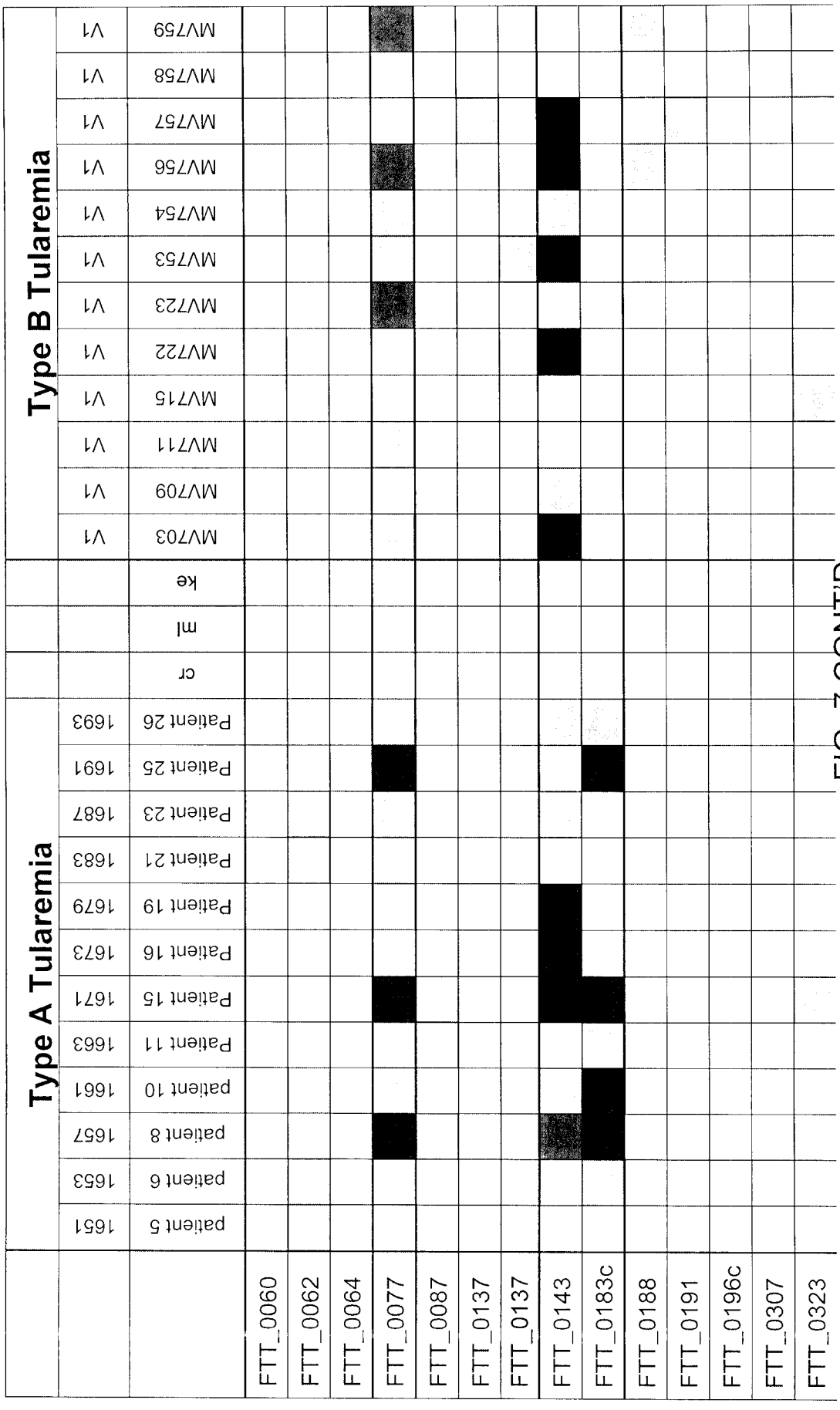

Type A Tularemia
Type B Tularemia
patient 5
patient 6
patient 8
patient 10
Patient 11
Patient 15
Patient 16
Patient 19
Patient 21
Patient 23
Patient 25
Patient 26
cr
ml
ke
MV703
MV709
MV711
MV715
MV722
MV723
MV753
MV754
MV756
MV757
MV758
MV759
V1
1651
1653
1657
1661
1663
1671
1673
1679
1683
1687
1691
1693
FTT_0060
FTT_0062
FTT_0064
FTT_0077
FTT_0087
FTT_0137
FTT_0137
FTT_0143
FTT_0183c
FTT_0188
FTT_0191
FTT_0196c
FTT_0307
FTT_0323

FIG. 7 CONT'D

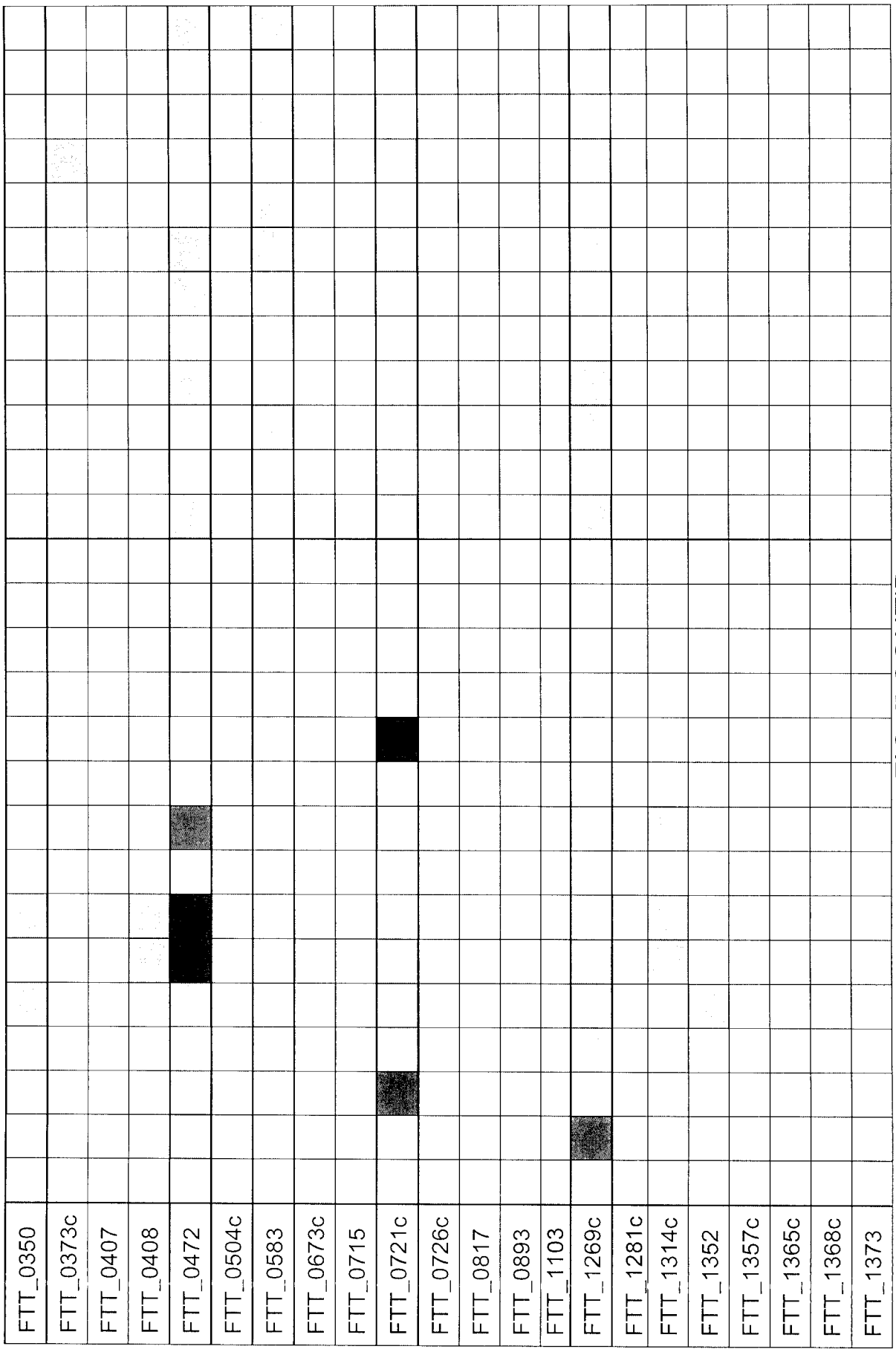

FTT_0350
FTT_0373c
FTT_0407
FTT_0408
FTT_0472
FTT_0504c
FTT_0583
FTT_0673c
FTT_0715
FTT_0721c
FTT_0726c
FTT_0817
FTT_0893
FTT_1103
FTT_1269c
FTT_1281c
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FTT_1352
FTT_1357c
FTT_1365c
FTT_1368c
FTT_1373

FIG. 7 CONT'D

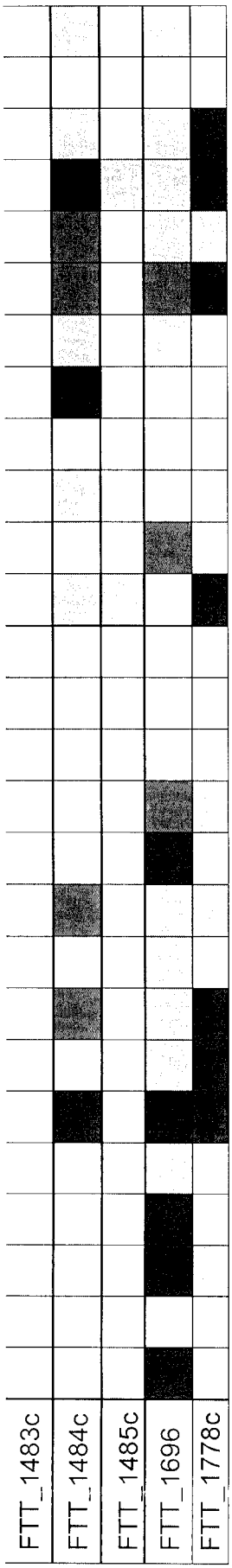


FIG. 7 CONT'D

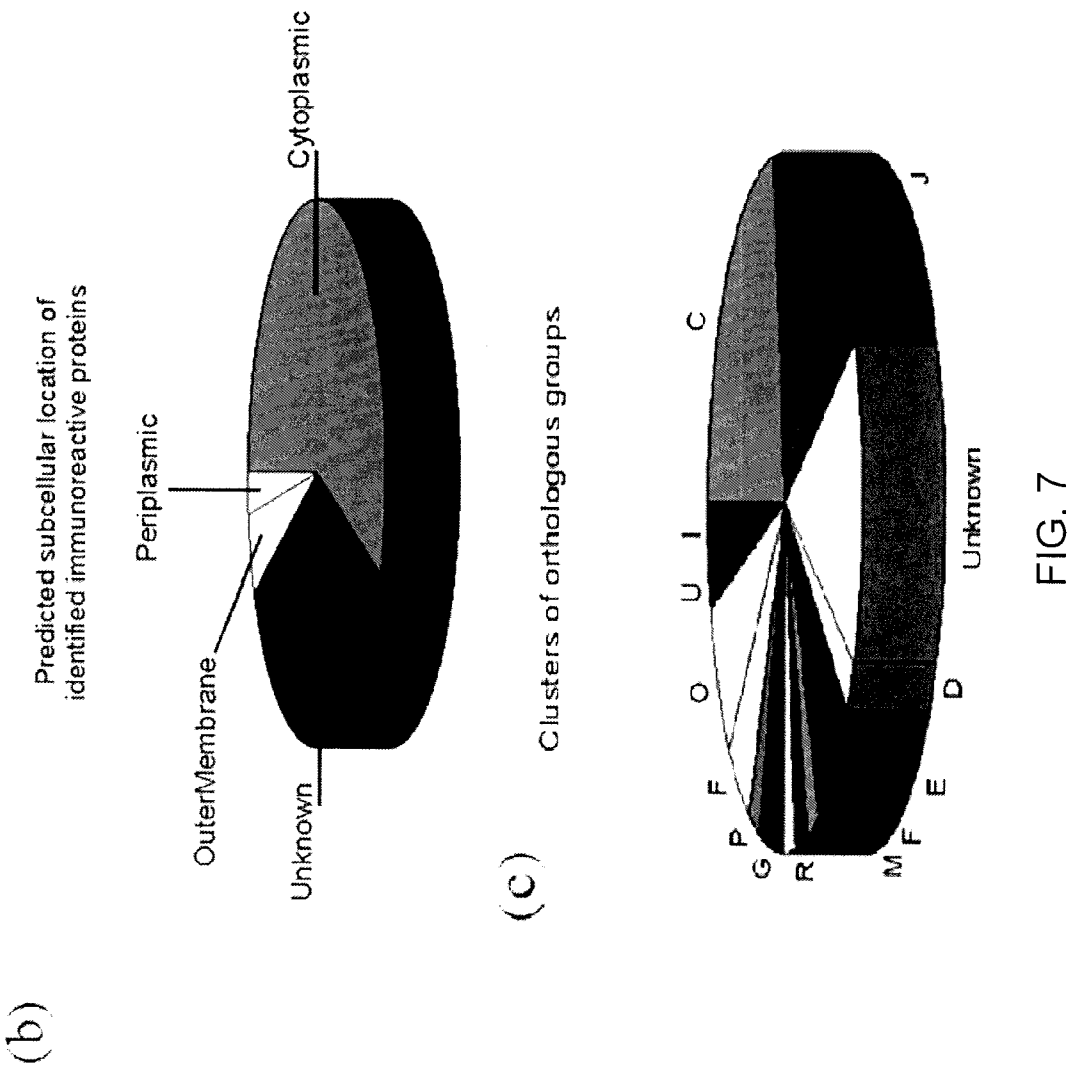

(b)
Predicted subcellular location of identified immunoreactive proteins
Cytoplasmic
Periplasmic
OuterMembrane
Unknown
(c)
Clusters of orthologous groups
J
C
I
U
O
F
P
G
R
M
F
E
D
Unknown

FIG. 7

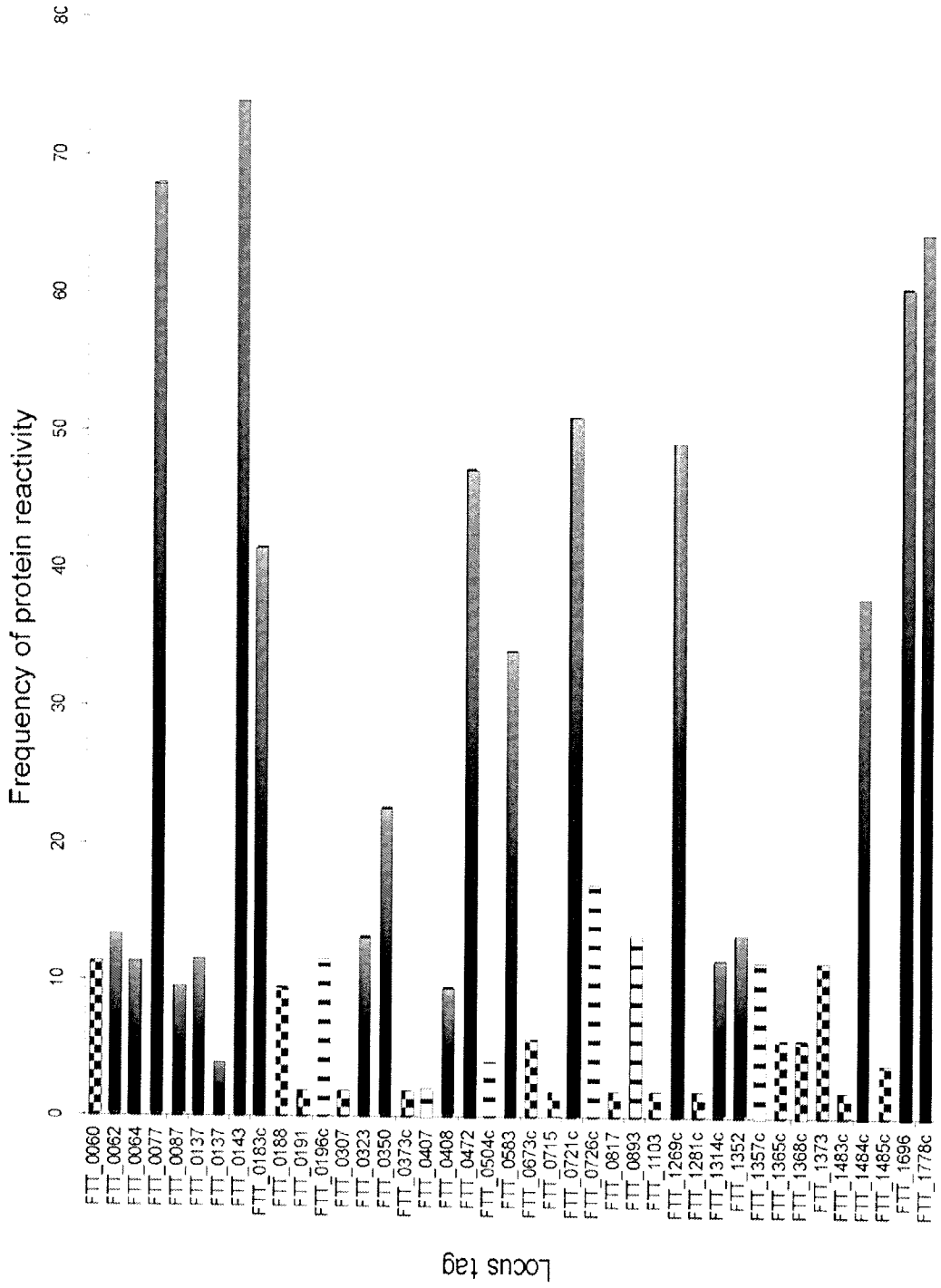

Frequency of protein reactivity
0
10
20
30
40
50
60
70
80
FTT_0060
FTT_0062
FTT_0064
FTT_0077
FTT_0087
FTT_0137
FTT_0137
FTT_0143
FTT_0183c
FTT_0188
FTT_0191
FTT_0196c
FTT_0307
FTT_0323
FTT_0350
FTT_0373c
FTT_0407
FTT_0408
FTT_0472
FTT_0504c
FTT_0583
FTT_0673c
FTT_0715
FTT_0721c
FTT_0726c
FTT_0817
FTT_0893
FTT_1103
FTT_1269c
FTT_1281c
FTT_1314c
FTT_1352
FTT_1357c
FTT_1365c
FTT_1368c
FTT_1373
FTT_1483c
FTT_1484c
FTT_1485c
FTT_1696
FTT_1778c
Locus tag

FIG. 8

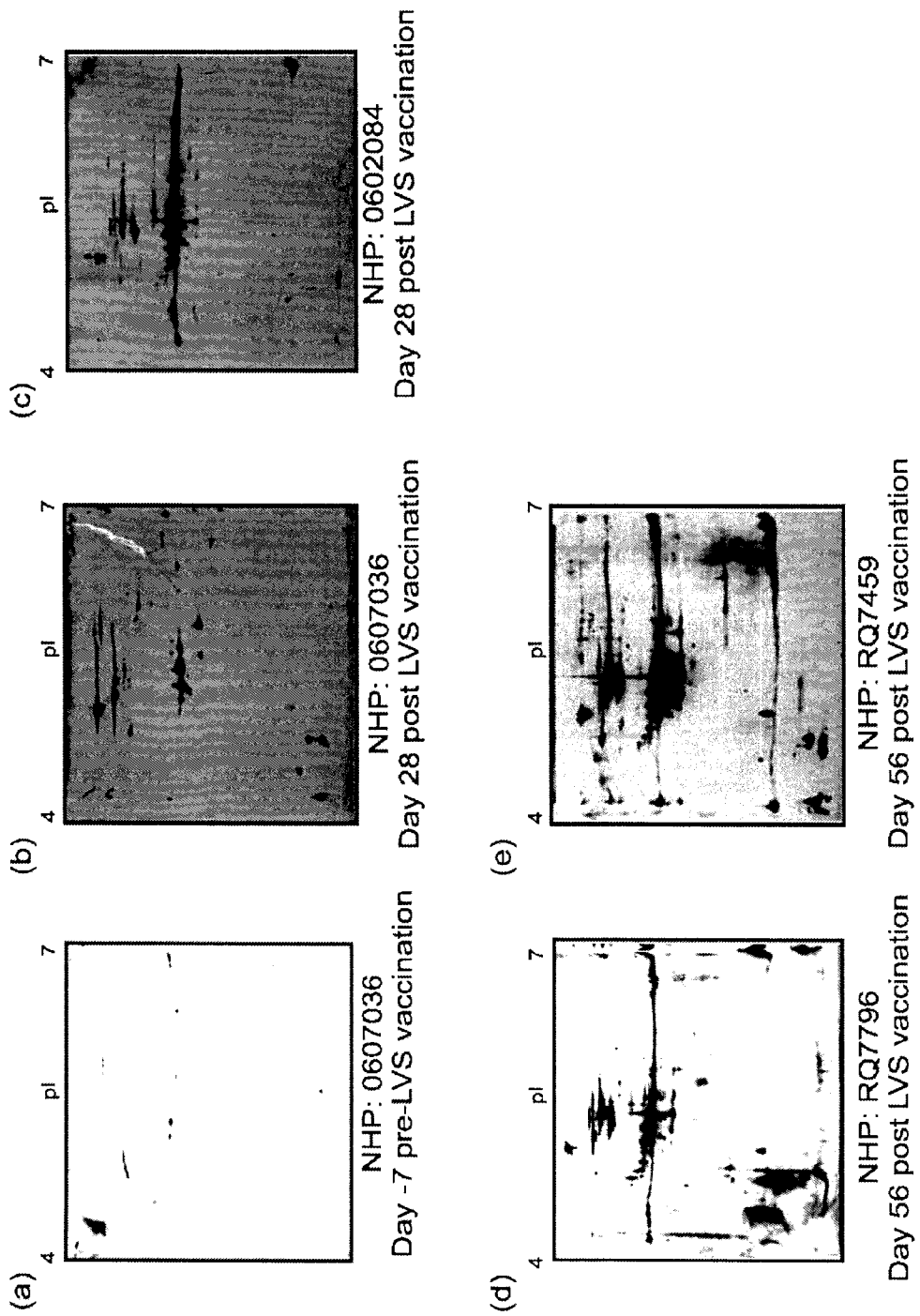

(a)
4
pI
7
NHP: 0607036
Day -7 pre-LVS vaccination
(b)
4
pI
7
NHP: 0607036
Day 28 post LVS vaccination
(c)
4
pI
7
NHP: 0602084
Day 28 post LVS vaccination
(d)
4
pI
7
NHP: RQ7796
Day 56 post LVS vaccination
(e)
4
pI
7
NHP: RQ7459
Day 56 post LVS vaccination

FIG. 9

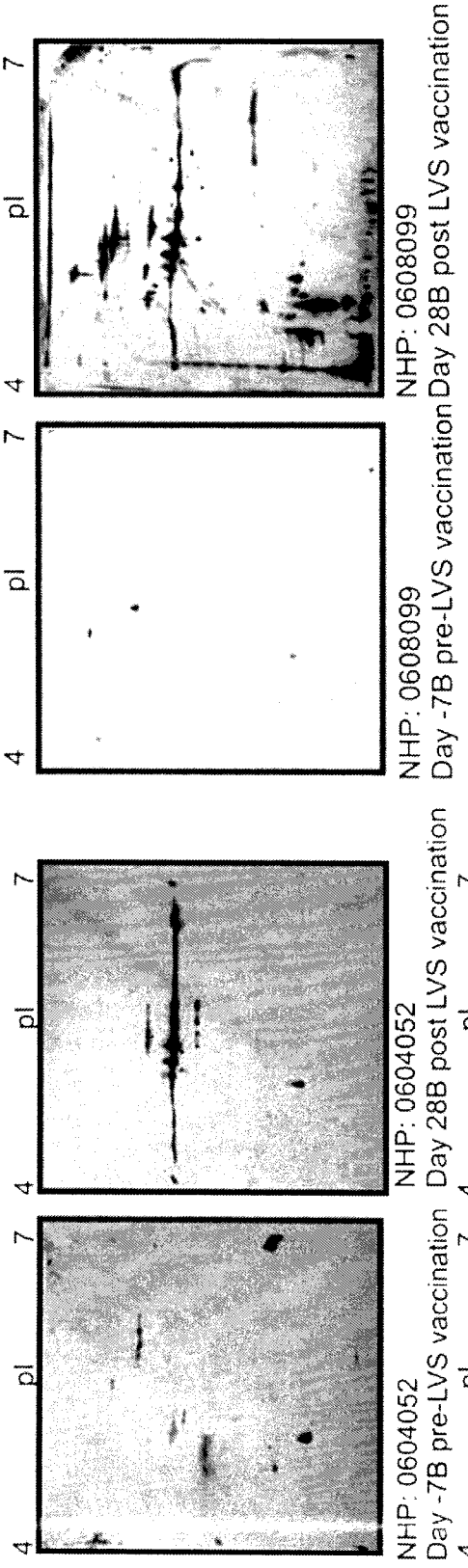

4
pI
7
NHP: 0604052
Day -7B pre-LVS vaccination
NHP: 0604052
Day 28B post LVS vaccination
NHP: 0608099
Day -7B pre-LVS vaccination
NHP: 0608099
Day 28B post LVS vaccination
NHP: RQ7477
Day-7A pre-LVS vaccination
NHP: RQ7477
Day 28A post LVS vaccination

FIG. 10A

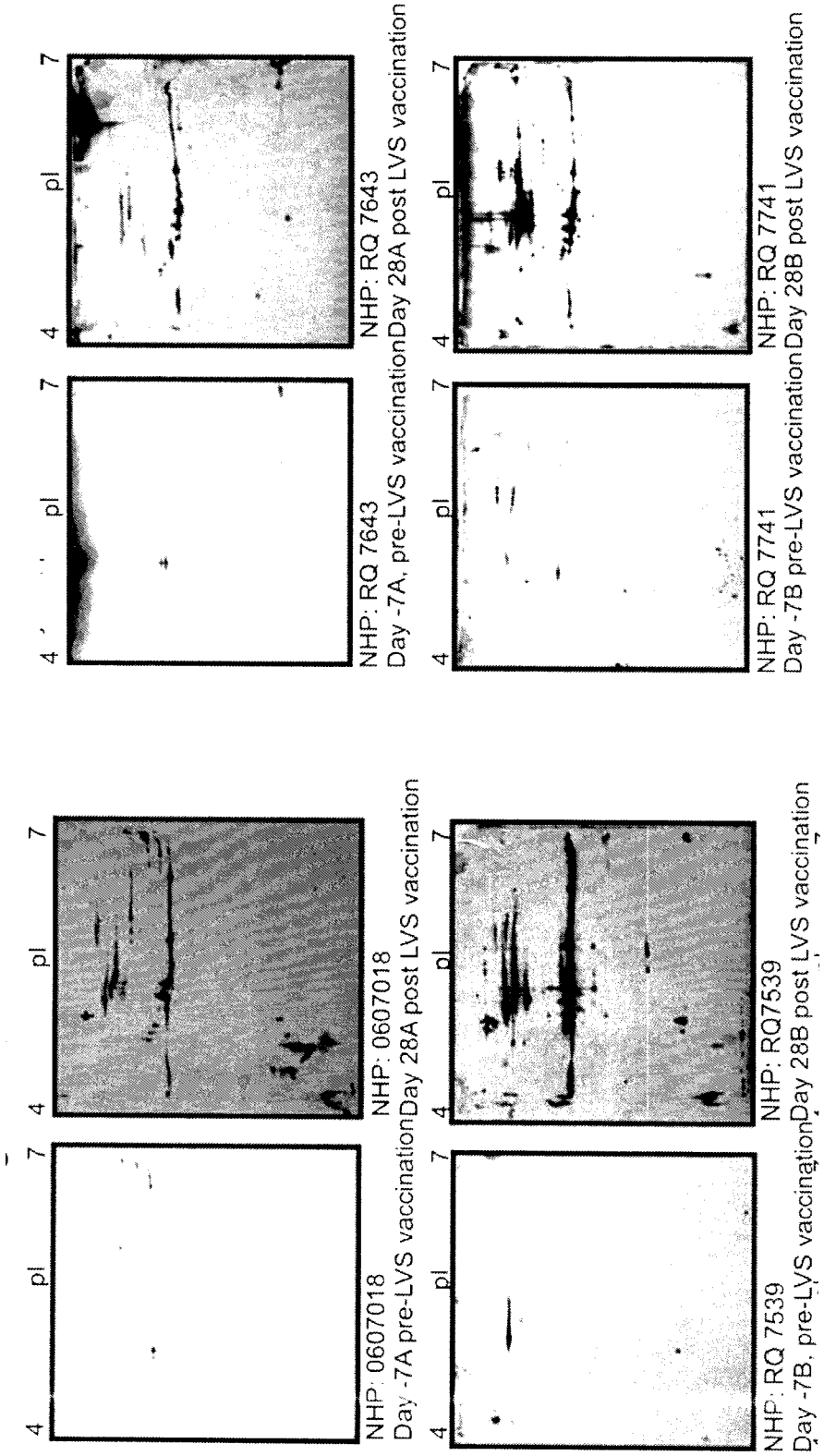

4
pl
7
NHP: 0607018
Day -7A pre-LVS vaccination
NHP: 0607018
Day 28A post LVS vaccination
NHP: RQ 7539
Day -7B, pre-LVS vaccination
NHP: RQ7539
Day 28B post LVS vaccination
NHP: RQ 7643
Day -7A, pre-LVS vaccination
NHP: RQ 7643
Day 28A post LVS vaccination
NHP: RQ 7741
Day -7B pre-LVS vaccination
NHP: RQ 7741
Day 28B post LVS vaccination

FIG. 10A

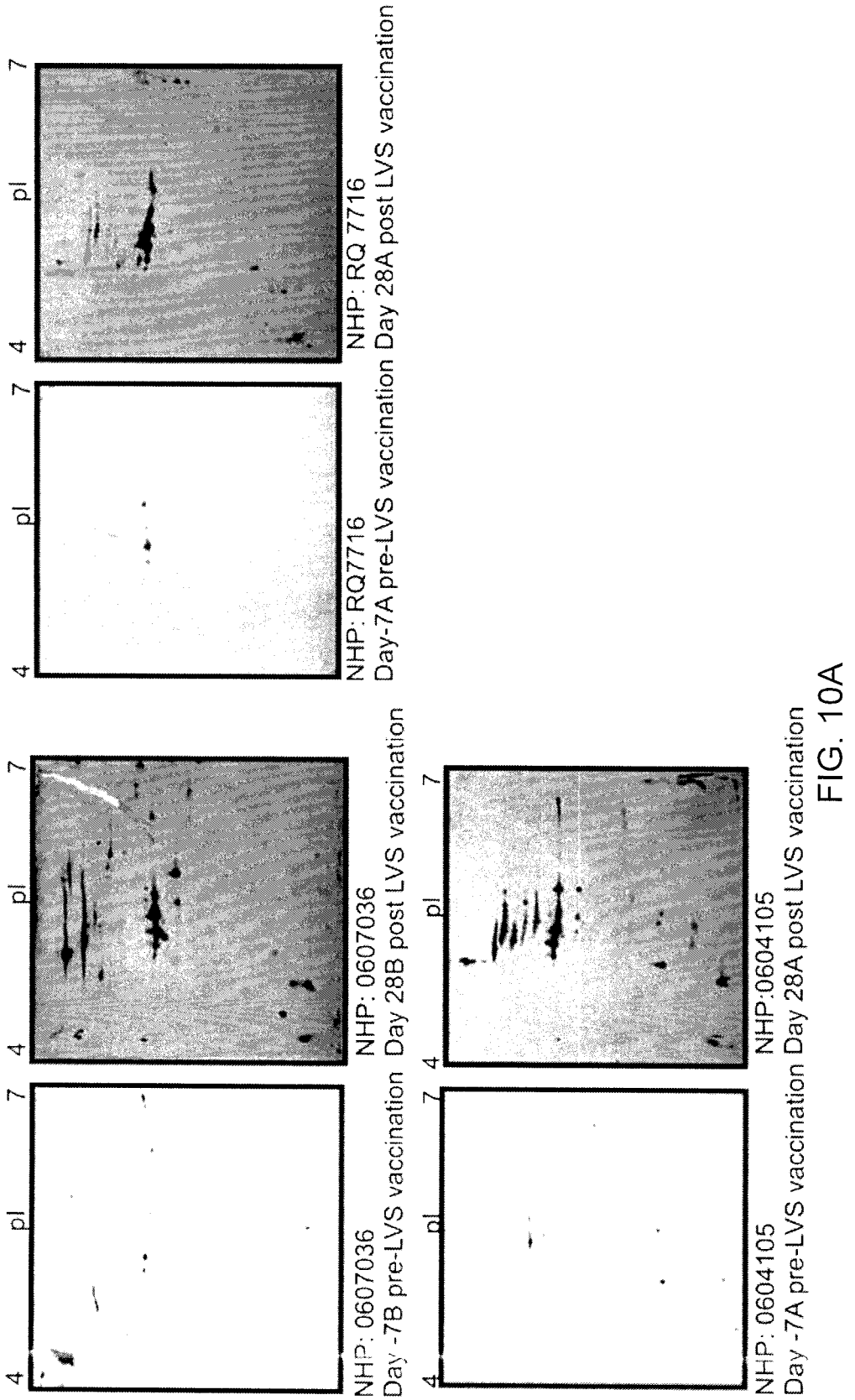

4
pl
7
NHP: 0607036
Day -7B pre-LVS vaccination
NHP: 0607036
Day 28B post LVS vaccination
NHP: RQ7716
Day-7A pre-LVS vaccination
NHP: RQ 7716
Day 28A post LVS vaccination
NHP: 0604105
Day -7A pre-LVS vaccination
NHP:0604105
Day 28A post LVS vaccination

FIG. 10A

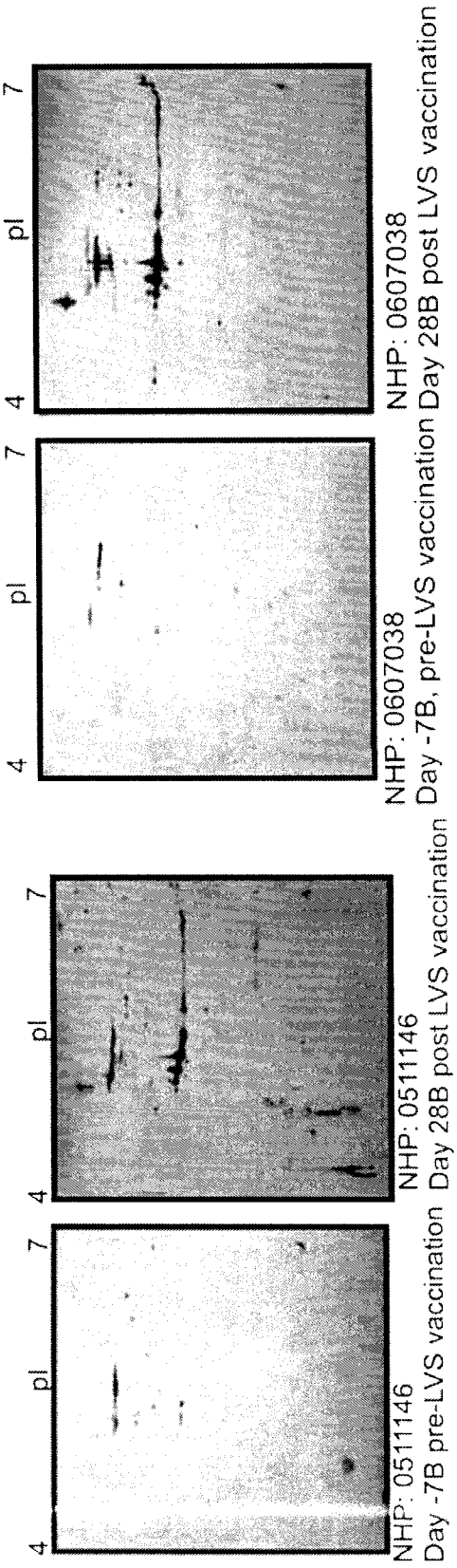

4
pI
7
NHP: 0511146
Day -7B pre-LVS vaccination
NHP: 0511146
Day 28B post LVS vaccination
NHP: 0602084
Day -7B pre-LVS vaccination
NHP: 0602084
Day 28B post LVS vaccination
NHP: 0607038
Day -7B, pre-LVS vaccination
NHP: 0607038
Day 28B post LVS vaccination

FIG. 10B

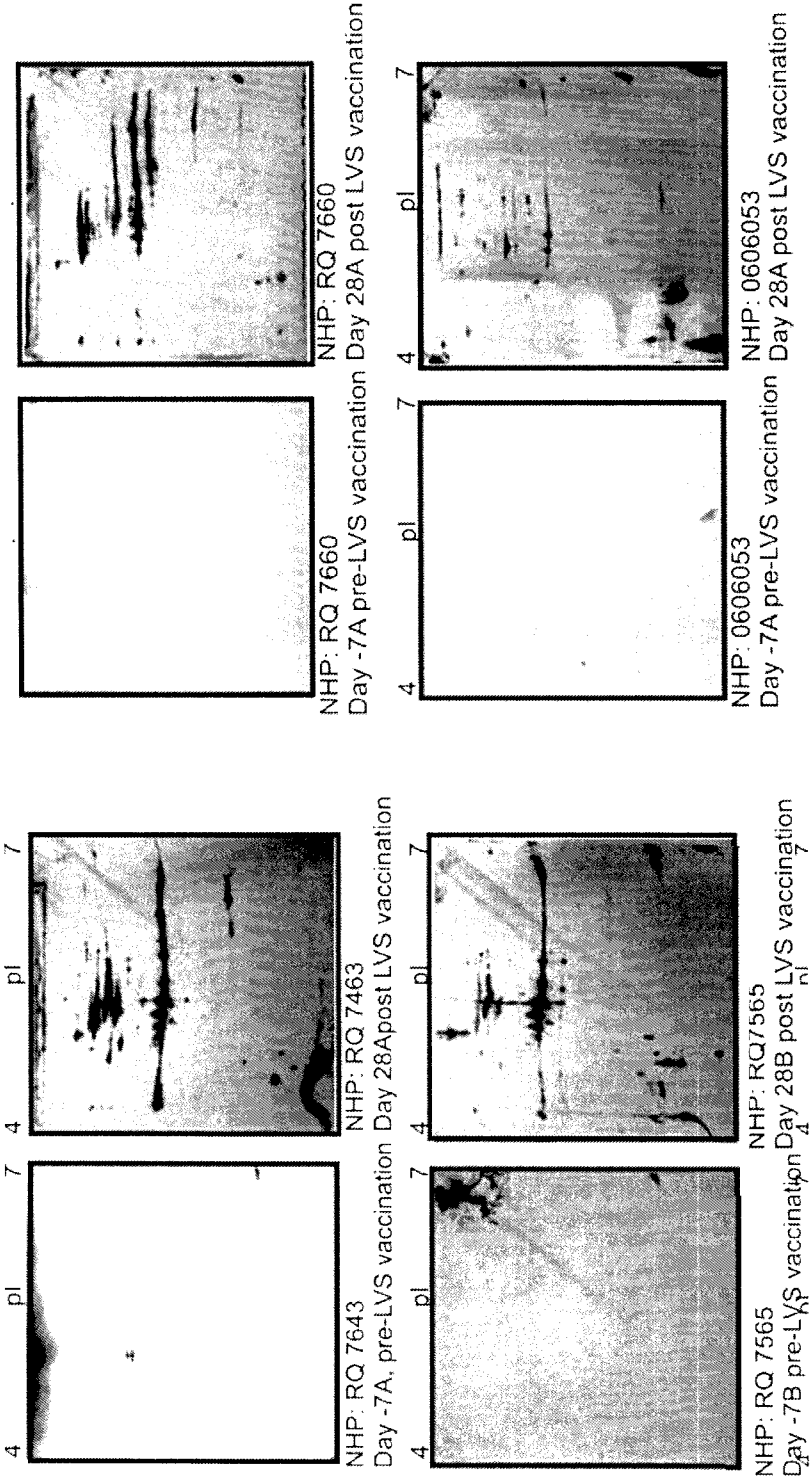

4
pI
7
NHP: RQ 7643
Day -7A, pre-LVS vaccination
NHP: RQ 7463
Day 28Apost LVS vaccination
NHP: RQ 7660
Day -7A pre-LVS vaccination
NHP: RQ 7660
Day 28A post LVS vaccination
NHP: RQ 7565
Day -7B pre-LVS vaccination
NHP: RQ7565
Day 28B post LVS vaccination
NHP: 0606053
Day -7A pre-LVS vaccination
NHP: 0606053
Day 28A post LVS vaccination

FIG. 10B

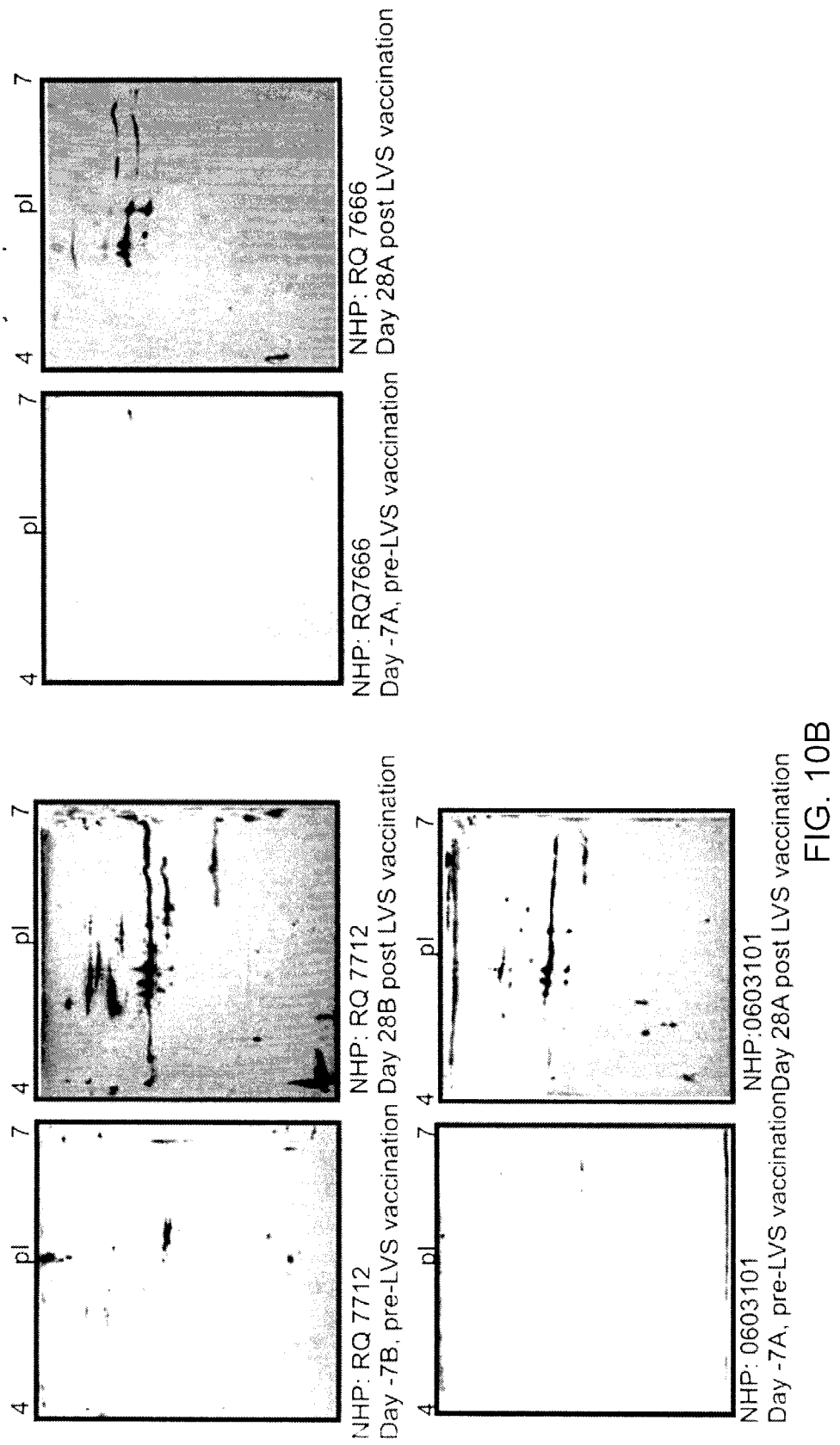

4
pI
7
NHP: RQ 7712
Day -7B, pre-LVS vaccination
NHP: RQ 7712
Day 28B post LVS vaccination
NHP:0603101
Day -7A, pre-LVS vaccination
NHP:0603101
Day 28A post LVS vaccination
NHP: RQ7666
Day -7A, pre-LVS vaccination
NHP: RQ 7666
Day 28A post LVS vaccination

FIG. 10B

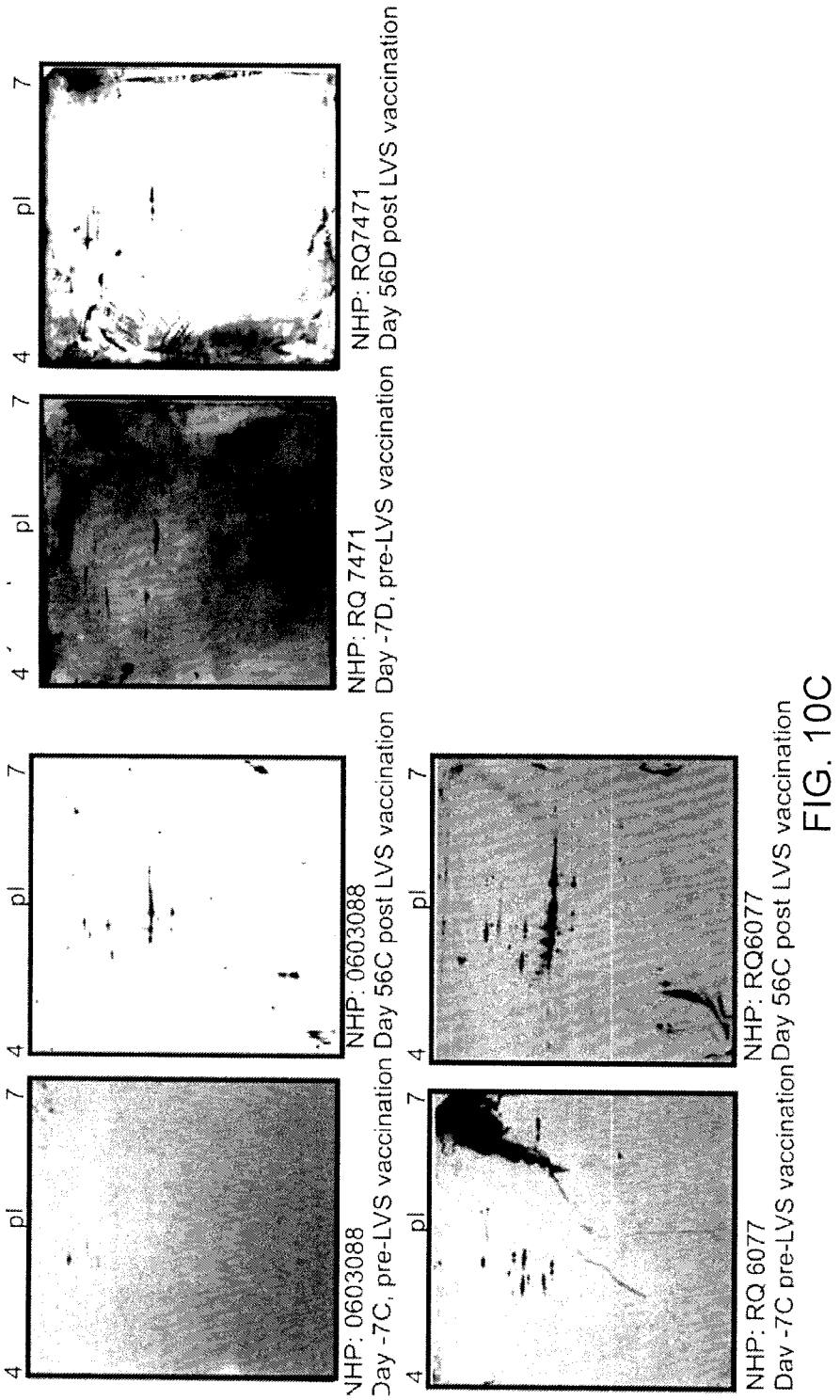

4
pl
7
NHP: 0603088
Day -7C, pre-LVS vaccination
NHP: 0603088
Day 56C post LVS vaccination
NHP: RQ 7471
Day -7D, pre-LVS vaccination
NHP: RQ7471
Day 56D post LVS vaccination
NHP: RQ 6077
Day -7C pre-LVS vaccination
NHP: RQ6077
Day 56C post LVS vaccination

FIG. 10C

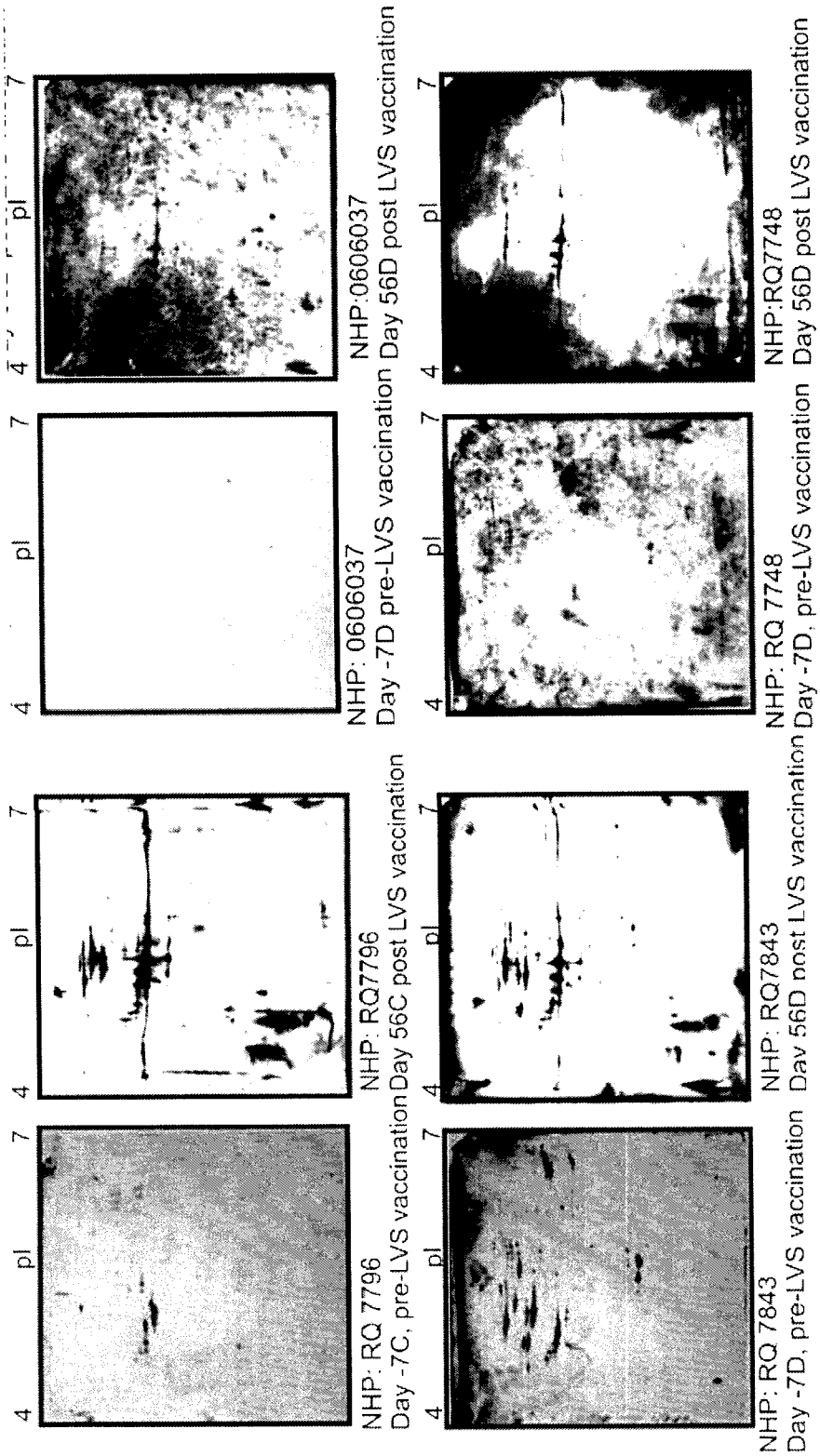

4
pl
7
NHP: RQ 7796
Day -7C, pre-LVS vaccination
NHP: RQ7796
Day 56C post LVS vaccination
NHP: 0606037
Day -7D pre-LVS vaccination
NHP:0606037
Day 56D post LVS vaccination
NHP: RQ 7843
Day -7D, pre-LVS vaccination
NHP: RQ7843
Day 56D post LVS vaccination
NHP: RQ 7748
Day -7D, pre-LVS vaccination
NHP:RQ7748
Day 56D post LVS vaccination

FIG. 10C

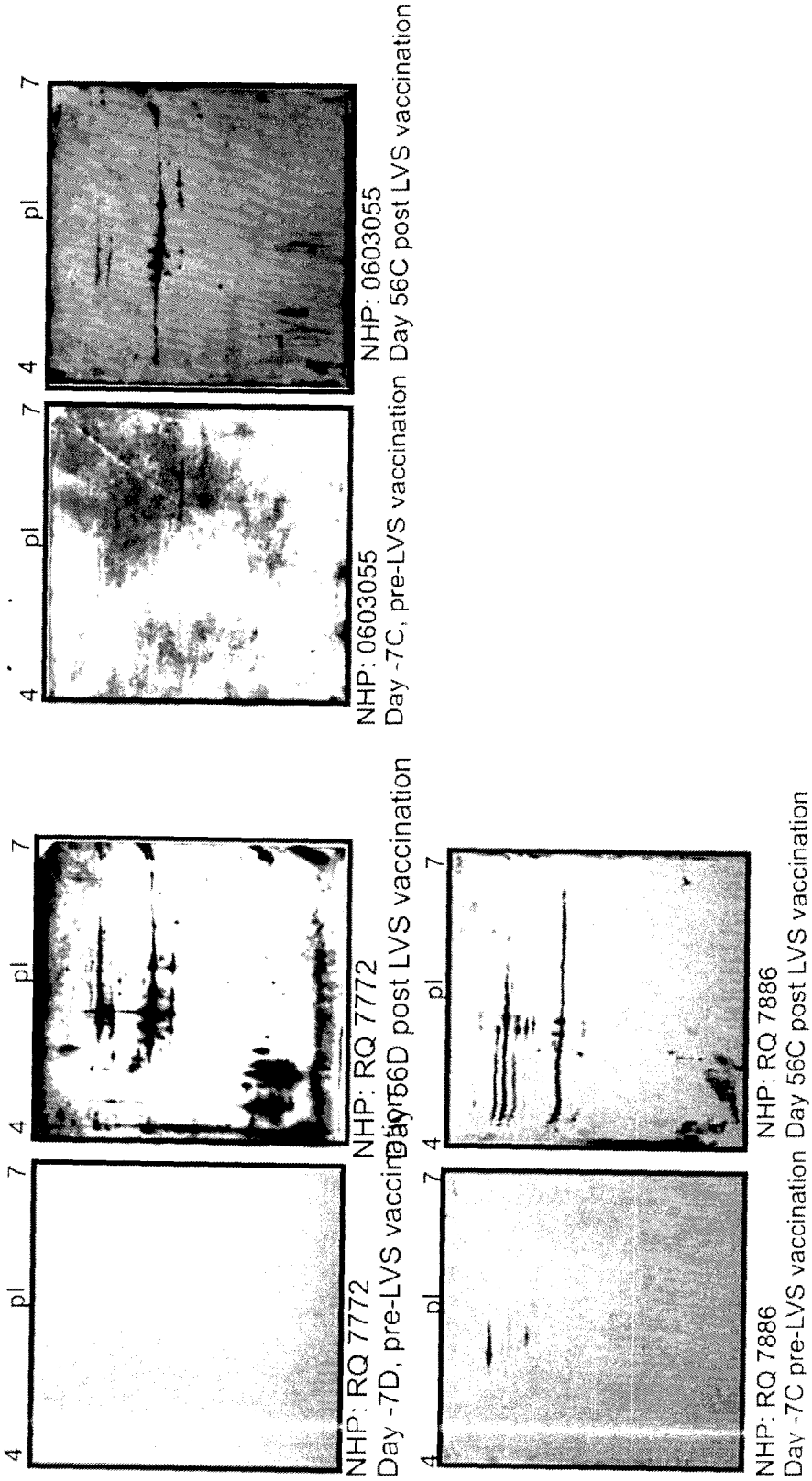

4
pl
7
NHP: RQ 7772
Day -7D, pre-LVS vaccination
NHP: RQ 7772
Day 56D post LVS vaccination
NHP: 0603055
Day -7C, pre-LVS vaccination
NHP: 0603055
Day 56C post LVS vaccination
NHP: RQ 7886
Day -7C pre-LVS vaccination
NHP: RQ 7886
Day 56C post LVS vaccination

FIG. 10C

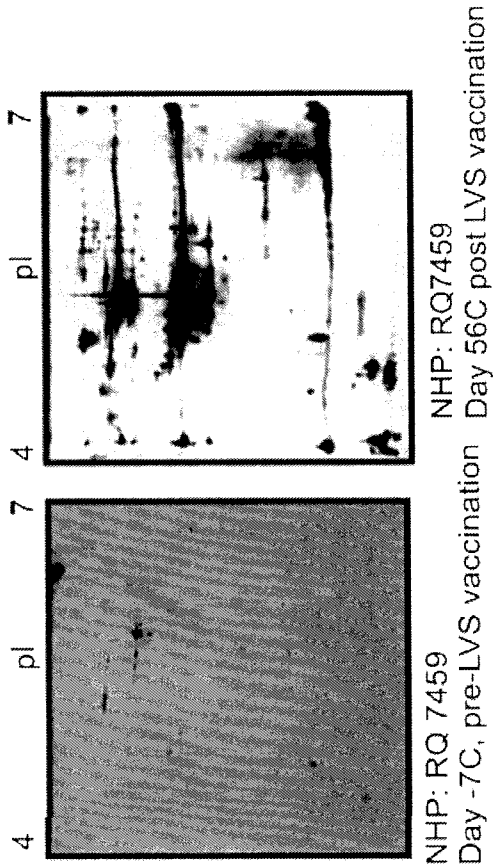

4
pI
7
NHP: 0607014
Day -7C, pre-LVS vaccination
NHP: 0607014
Day 56C post LVS vaccination
NHP: RQ 7459
Day -7C, pre-LVS vaccination
NHP: RQ7459
Day 56C post LVS vaccination
NHP: RQ 7139
Day -7C, pre-LVS vaccination
NHP: RQ7139
Day 56C post LVS vaccination

FIG. 10D

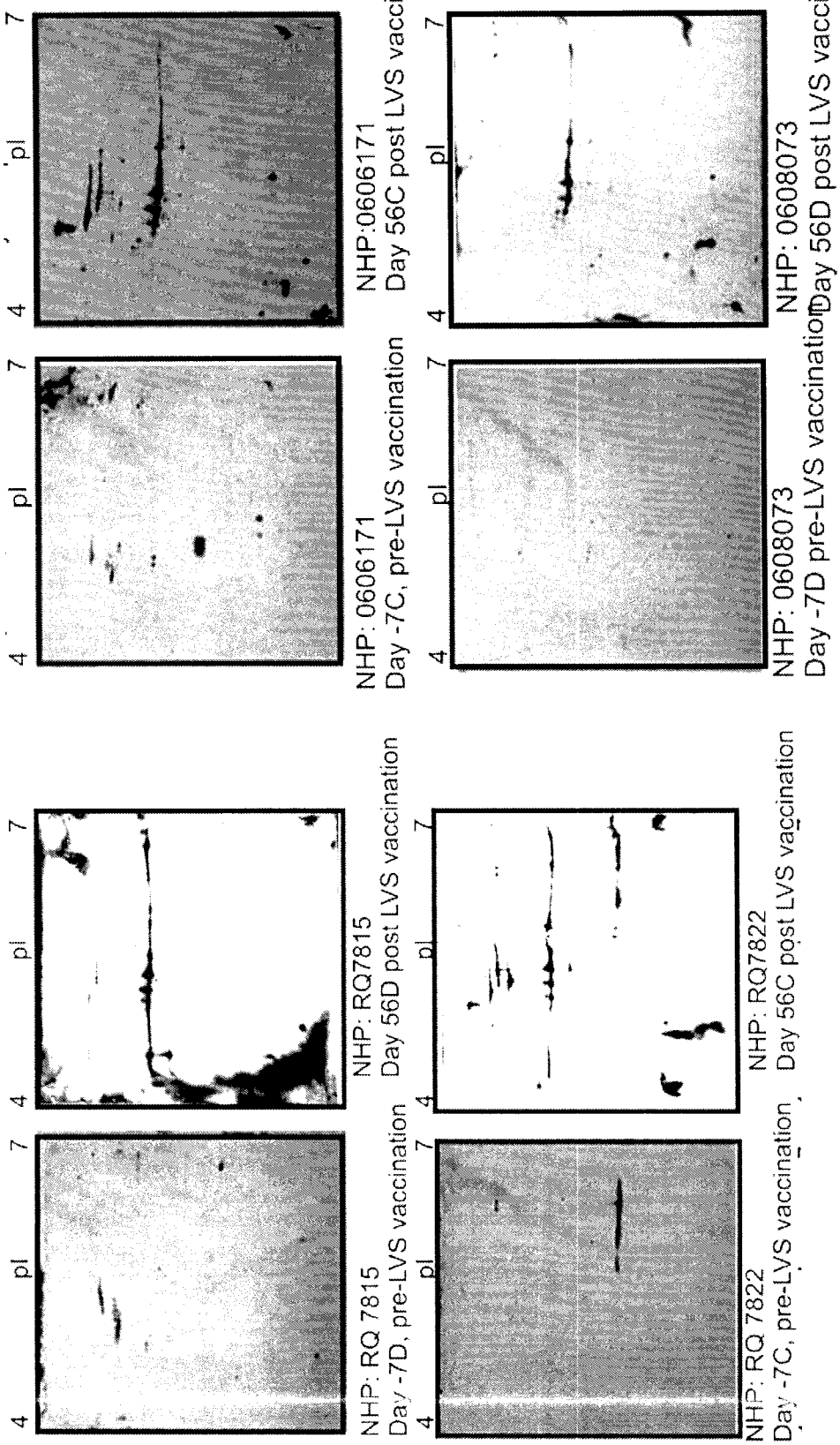

4
pl
7
NHP: RQ 7815
Day -7D, pre-LVS vaccination
NHP: RQ7815
Day 56D post LVS vaccination
NHP: RQ 7822
Day -7C, pre-LVS vaccination
NHP: RQ7822
Day 56C post LVS vaccination
NHP: 0606171
Day -7C, pre-LVS vaccination
NHP:0606171
Day 56C post LVS vaccination
NHP: 0608073
Day -7D pre-LVS vaccination
NHP: 0608073
Day 56D post LVS vaccination

FIG. 10D

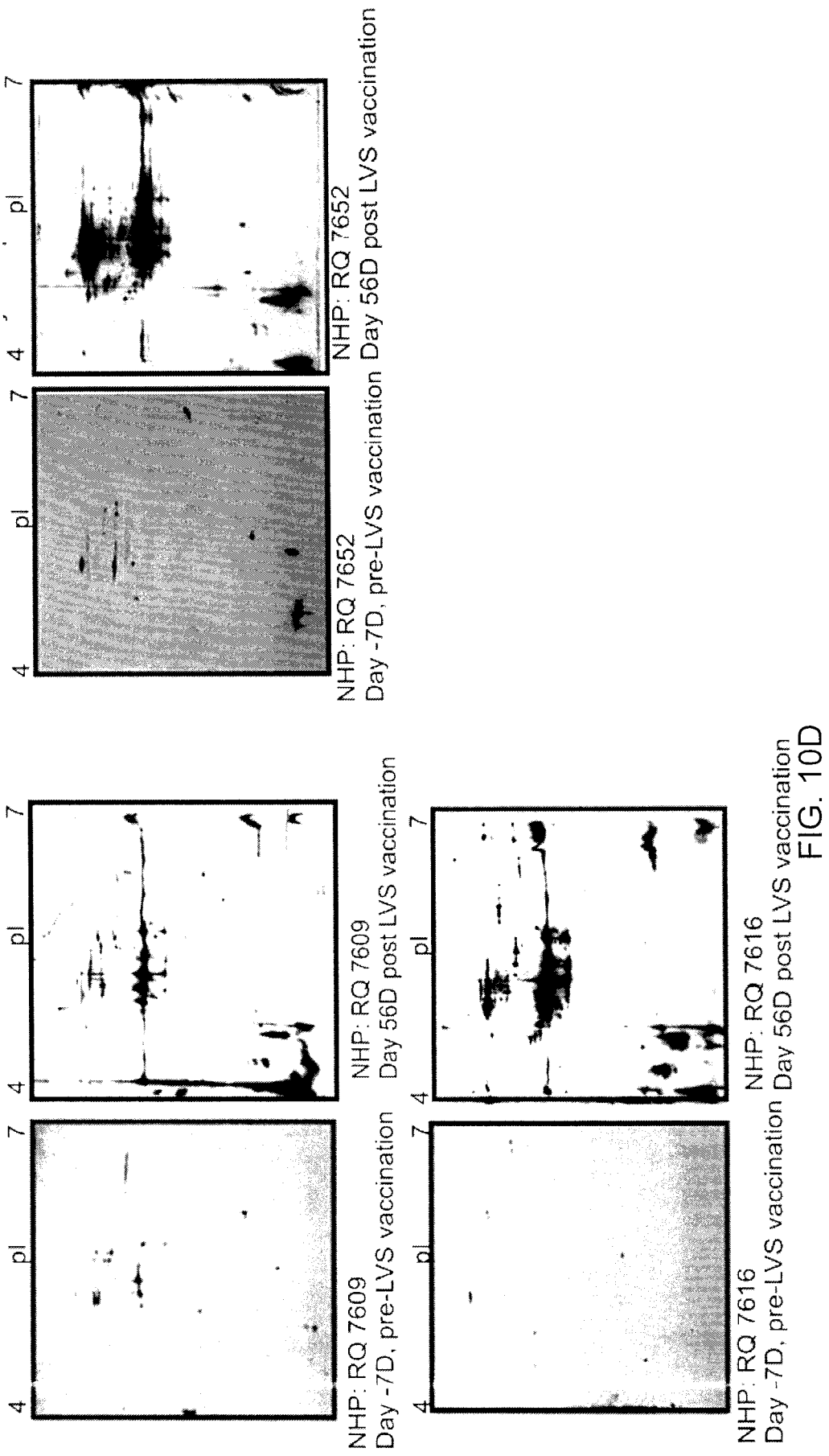

4
pI
7
NHP: RQ 7652
Day -7D, pre-LVS vaccination
NHP: RQ 7652
Day 56D post LVS vaccination
NHP: RQ 7609
Day -7D, pre-LVS vaccination
NHP: RQ 7609
Day 56D post LVS vaccination
NHP: RQ 7616
Day -7D, pre-LVS vaccination
NHP: RQ 7616
Day 56D post LVS vaccination

FIG. 10D

Group 3, Scarification, Challenge Day 36																					
		0604052	Day 28B	0607018	Day 28A	0607036	Day 28B	RQ7477	Day 28A	RQ7539	Day 28B	0604105	Day 28A	0608099	Day 28B	RQ7643	Day 28A	RQ7716	Day 28A	RQ7741	Day 28B
LOCUS TAG	Protein identification																				
FTT_0062	ATP synthase subunit A																				
FTT_0064	ATP synthase subunit B																				
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase; 30S ribosomal protein S1																				
FTT_0137	Elongation factor Tu (EF-Tu)																				
FTT_0188	Cell division protein																				
FTT_0323	Elongation factor G (EF-G)																				
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit																				
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit																				
FTT_0715	Chitinase family 18 protein																				
FTT_0721c	Peroxidase/catalase																				
FTT_0890c	Type IV pili fiber building block protein																				
FTT_1103	conserved hypothetical lipoprotein																				
FTT_1269c	Chaperone protein dnaK																				
FTT_1357c	intracellular growth locus, subunit C																				
FTT_1358c	intracellular growth locus, subunit B																				
FTT_1484c	Pyruvate dehydrogenase, E2 component																				
FTT_1696	Chaperone protein, groEL																				
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*																				
FTT_1374	malonyl coA-acyl carrier protein transacylase																				
FTT_0087	Aconitate Hydratase (Unidentified 1)																				
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)																				
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)																				
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)																				
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)																				
FTT_1155c	shikimate kinase I (Unidentified 8)																				
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)																				

FIG. 11

Group 4, Subcutaneous, Challenge Day 36		Day 28B	Day 28B	Day 28B	Day 28A	Day 28B	Day 28A	Day 28A	Day 28A	Day 28A	Day 28B
LOCUS TAG	Protein identification	0511146	0602084	0607038	RQ7463	RQ7565	0603101	0606053	RQ7660	RQ7666	RQ7712
FTT_0062	ATP synthase subunit A										
FTT_0064	ATP synthase subunit B										
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase; 30S ribosomal protein S1										
FTT_0137	Elongation factor Tu (EF-Tu)										
FTT_0188	Cell division protein										
FTT_0323	Elongation factor G (EF-G)										
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit										
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit										
FTT_0715	Chitinase family 18 protein										
FTT_0721c	Peroxidase/catalase										
FTT_0890c	Type IV pili fiber building block protein										
FTT_1103	conserved hypothetical lipoprotein										
FTT_1269c	Chaperone protein dnaK										
FTT_1357c	intracellular growth locus, subunit C										
FTT_1358c	intracellular growth locus, subunit B										
FTT_1484c	Pyruvate dehydrogenase, E2 component										
FTT_1696	Chaperone protein, groEL										
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*										
FTT_1374	malonyl coA-acyl carrier protein transacylase										
FTT_0087	Aconitate Hydratase (Unidentified 1)										
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)										
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)										
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)										
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)										
FTT_1155c	shikimate kinase I (Unidentified 8)										
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)										

FIG. 11 (CONT'D)

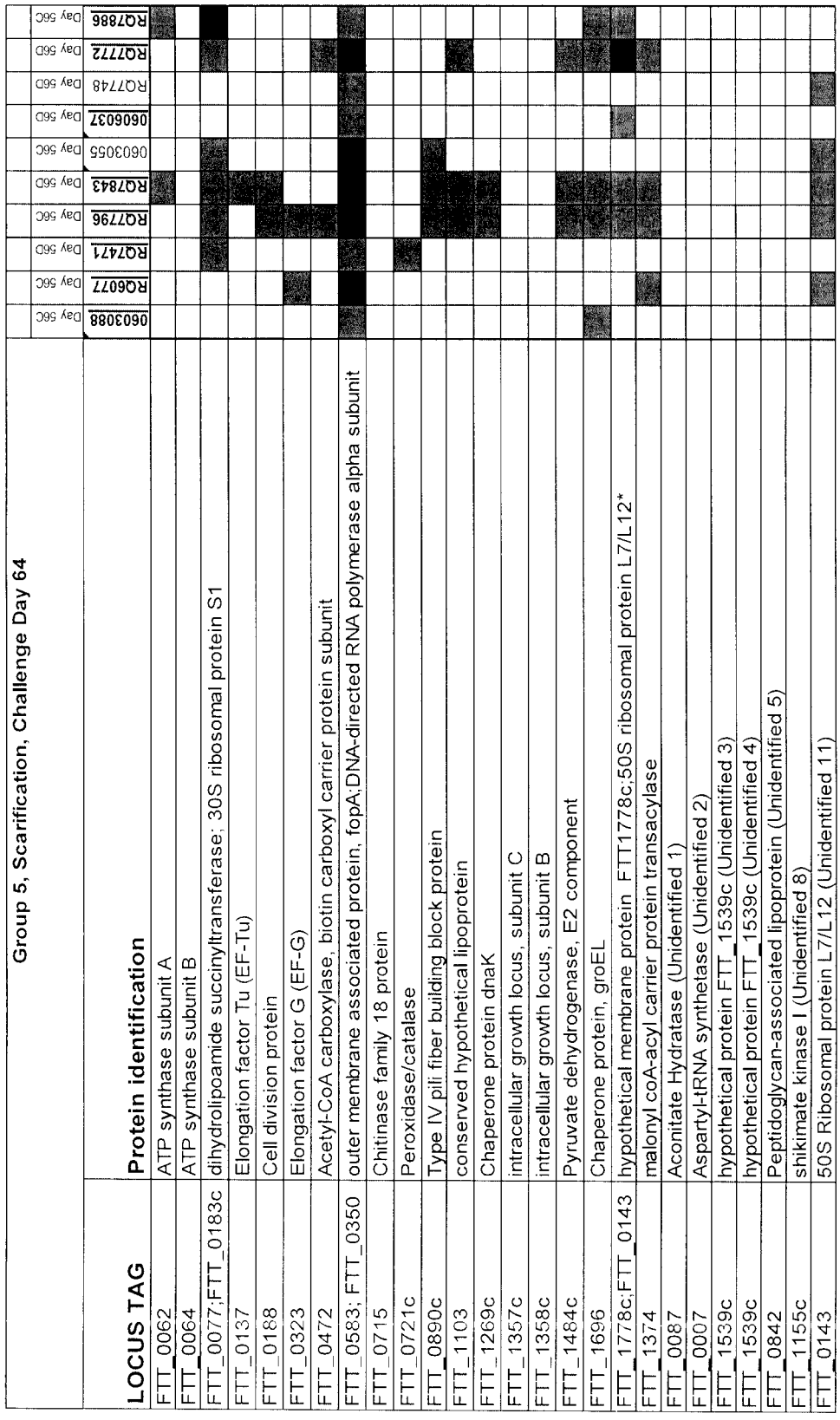

Group 5, Scarification, Challenge Day 64
LOCUS TAG
Protein identification
0603088 Day 56C
RQ6077 Day 56C
RQ7471 Day 56C
RQ7796 Day 56C
RQ7843 Day 56C
0603055 Day 56C
0606037 Day 56C
RQ7748 Day 56C
RQ7772 Day 56C
RQ7886 Day 56C
FTT_0062 ATP synthase subunit A
FTT_0064 ATP synthase subunit B
FTT_0077;FTT_0183c dihydrolipoamide succinyltransferase; 30S ribosomal protein S1
FTT_0137 Elongation factor Tu (EF-Tu)
FTT_0188 Cell division protein
FTT_0323 Elongation factor G (EF-G)
FTT_0472 Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit
FTT_0583; FTT_0350 outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit
FTT_0715 Chitinase family 18 protein
FTT_0721c Peroxidase/catalase
FTT_0890c Type IV pili fiber building block protein
FTT_1103 conserved hypothetical lipoprotein
FTT_1269c Chaperone protein dnaK
FTT_1357c intracellular growth locus, subunit C
FTT_1358c intracellular growth locus, subunit B
FTT_1484c Pyruvate dehydrogenase, E2 component
FTT_1696 Chaperone protein, groEL
FTT_1778c;FTT_0143 hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*
FTT_1374 malonyl coA-acyl carrier protein transacylase
FTT_0087 Aconitate Hydratase (Unidentified 1)
FTT_0007 AspartyI-tRNA synthetase (Unidentified 2)
FTT_1539c hypothetical protein FTT_1539c (Unidentified 3)
FTT_1539c hypothetical protein FTT_1539c (Unidentified 4)
FTT_0842 Peptidoglycan-associated lipoprotein (Unidentified 5)
FTT_1155c shikimate kinase I (Unidentified 8)
FTT_0143 50S Ribosomal protein L7/L12 (Unidentified 11)

FIG. 11 (CONT'D)

Group 6, Subcutaneous, Challenge Day 64												
			Day 56C	Day 56C	Day 56C	Day 56D	Day 56C	Day 56C	Day 56C	Day 56D	Day 56D	Day 56D
LOCUS TAG	Protein identification		0607014	RQ7139	RQ7459	RQ7815	RQ7822	0606171	0608073	RQ7609	RQ7616	RQ7652
FTT_0062	ATP synthase subunit A											
FTT_0064	ATP synthase subunit B											
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase; 30S ribosomal protein S1											
FTT_0137	Elongation factor Tu (EF-Tu)											
FTT_0188	Cell division protein											
FTT_0323	Elongation factor G (EF-G)											
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit											
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit											
FTT_0715	Chitinase family 18 protein											
FTT_0721c	Peroxidase/catalase											
FTT_0890c	Type IV pili fiber building block protein											
FTT_1103	conserved hypothetical lipoprotein											
FTT_1269c	Chaperone protein dnaK											
FTT_1357c	intracellular growth locus, subunit C											
FTT_1358c	intracellular growth locus, subunit B											
FTT_1484c	Pyruvate dehydrogenase, E2 component											
FTT_1696	Chaperone protein, groEL											
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*											
FTT_1374	malonyl coA-acyl carrier protein transacylase											
FTT_0087	Aconitate Hydratase (Unidentified 1)											
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)											
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)											
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)											
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)											
FTT_1155c	shikimate kinase I (Unidentified 8)											
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)											

FIG. 11 (CONT'D)

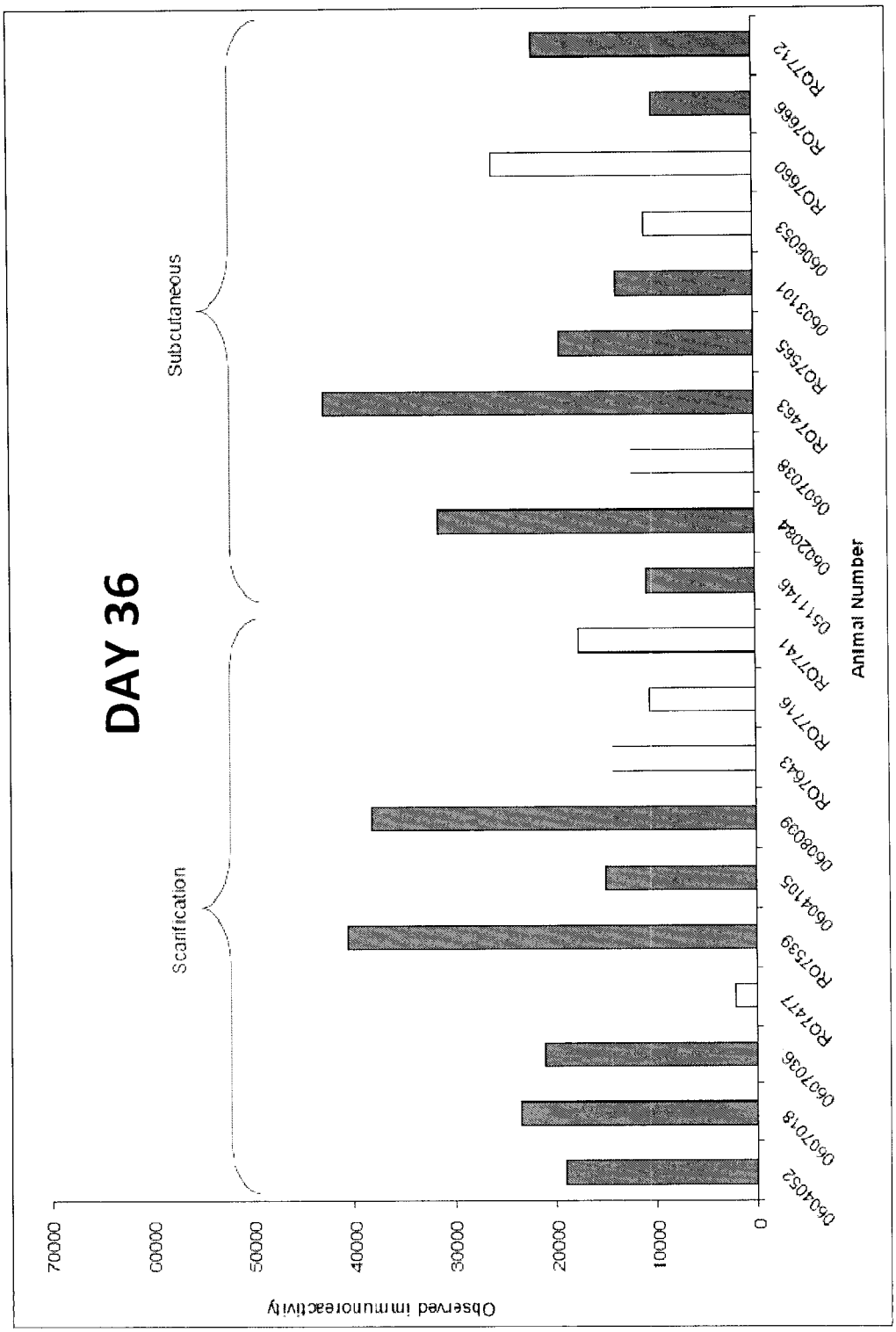

DAY 36
Scarification
Subcutaneous
Observed immunoreactivity
70000
60000
50000
40000
30000
20000
10000
0
Animal Number
060402
060701
060703
RO7427
RO7539
060410
060803
RO7643
RO7716
RO7741
051146
060208
060703
RO7463
RO7565
060310
060605
RO7660
RO7666
RO7712

FIG. 12

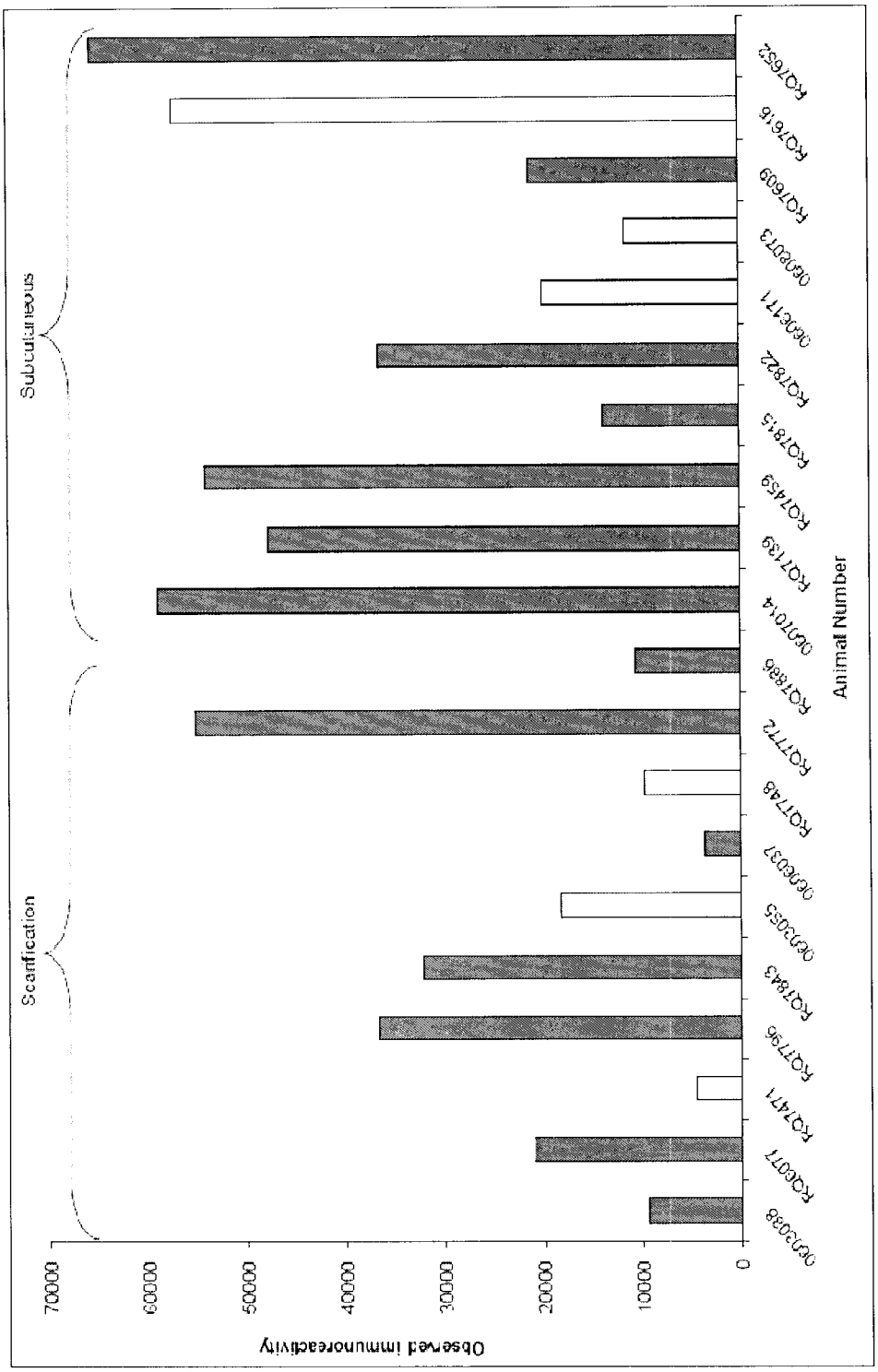

Observed Immunoreactivity
70000
60000
50000
40000
30000
20000
10000
0
Animal Number
Scarification
Subcutaneous

FIG. 12 (CONT'D)

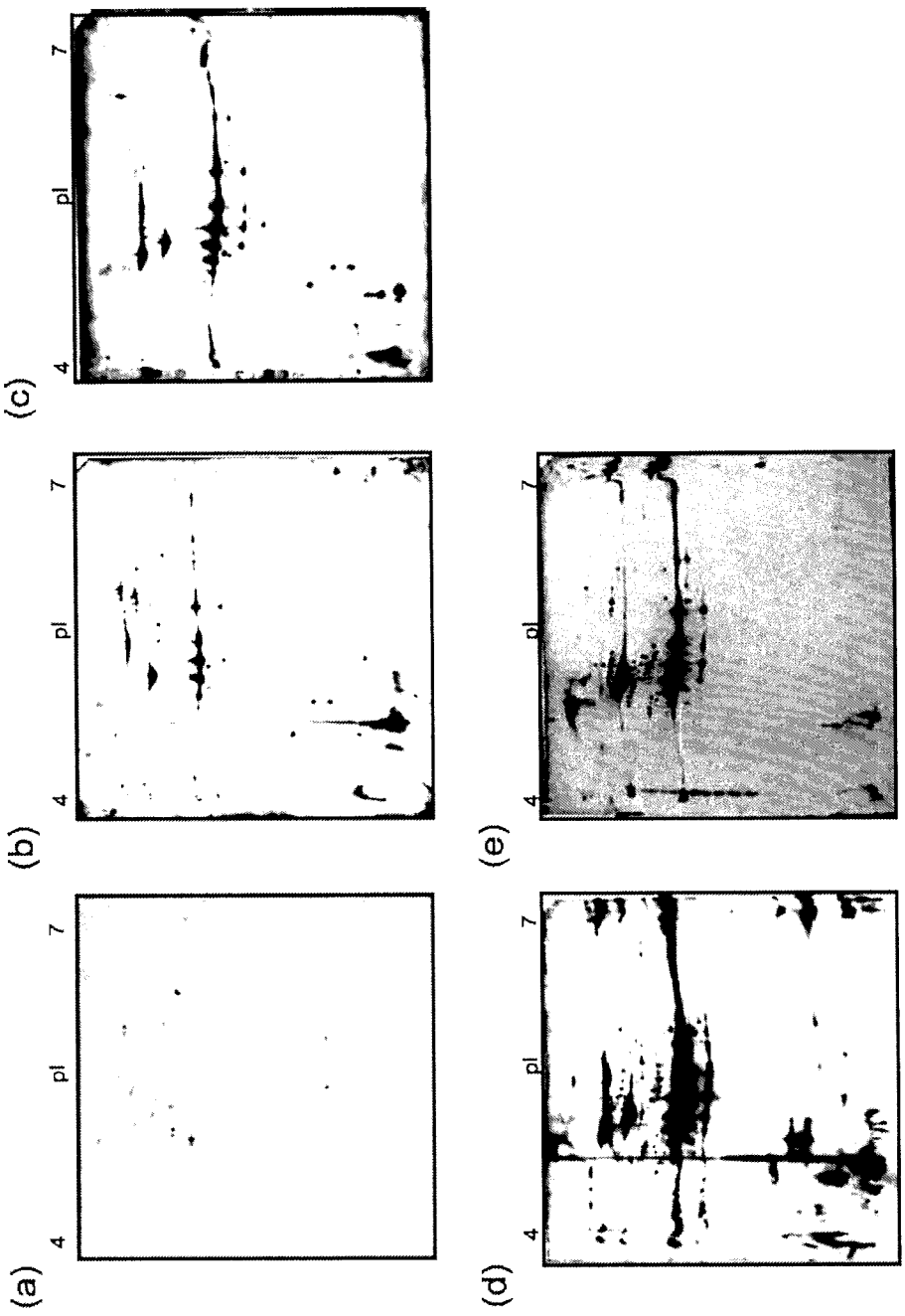


FIG. 13

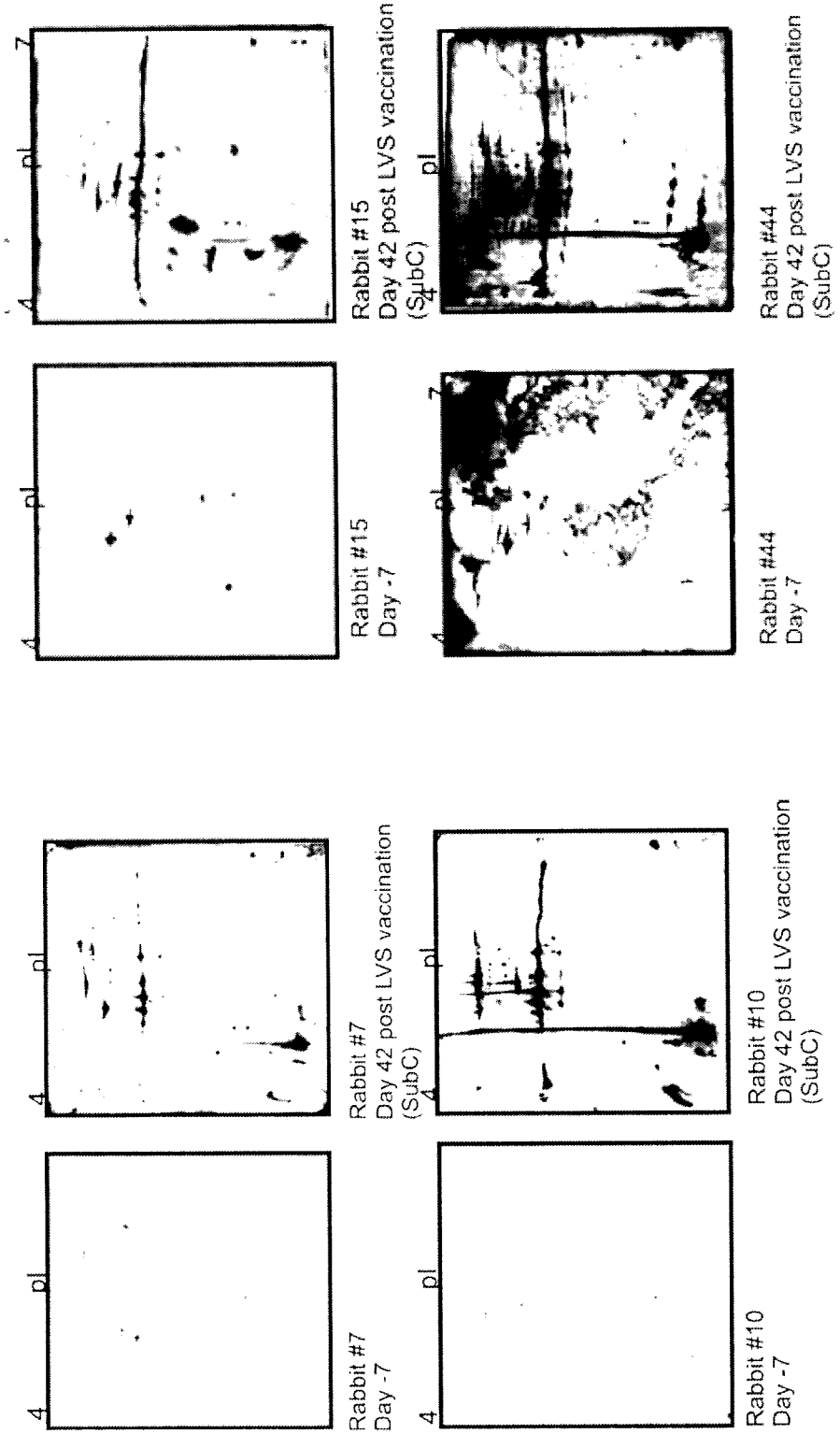

Rabbit #7
Day -7
Rabbit #7
Day 42 post LVS vaccination
(SubC)
Rabbit #10
Day -7
Rabbit #10
Day 42 post LVS vaccination
(SubC)
Rabbit #15
Day -7
Rabbit #15
Day 42 post LVS vaccination
(SubC)
Rabbit #44
Day -7
Rabbit #44
Day 42 post LVS vaccination
(SubC)
pI
4
7

FIG. 14A

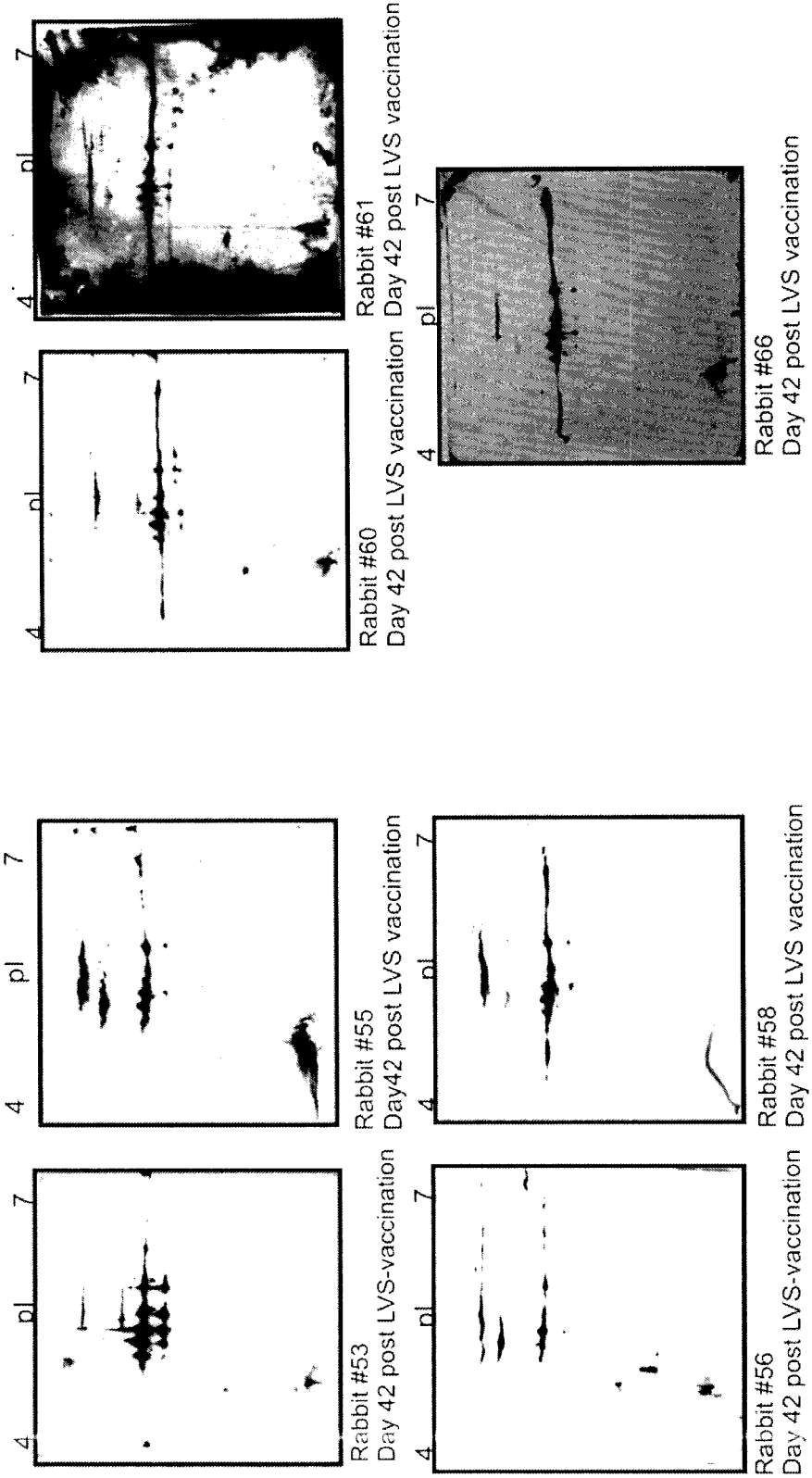
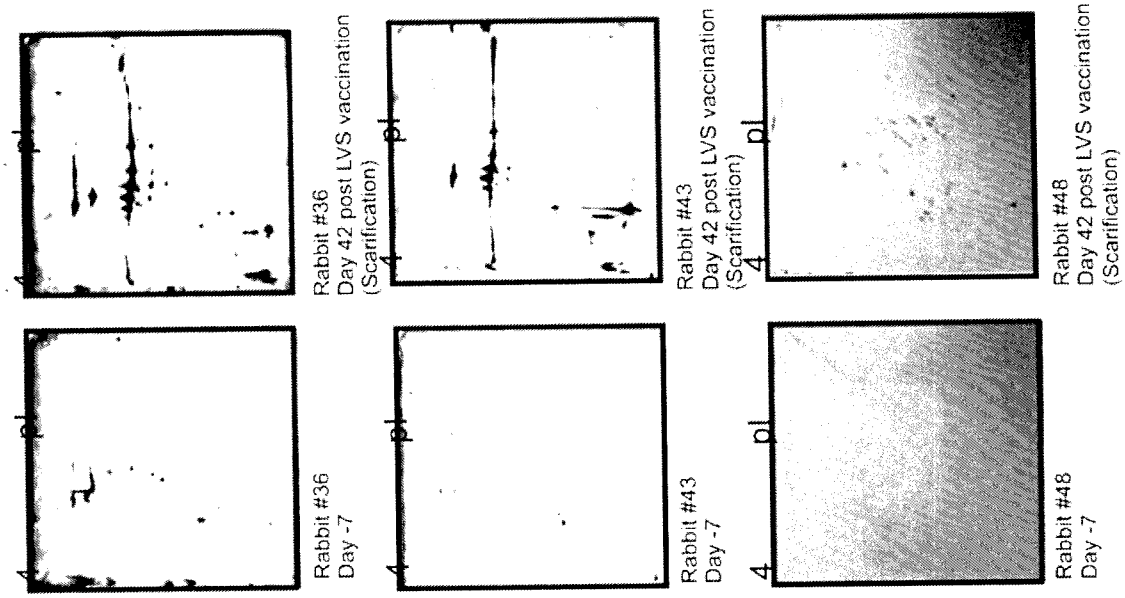

4
pl
7
Rabbit #53
Day 42 post LVS-vaccination
Rabbit #55
Day42 post LVS vaccination
Rabbit #56
Day 42 post LVS-vaccination
Rabbit #58
Day 42 post LVS vaccination
Rabbit #60
Day 42 post LVS vaccination
Rabbit #61
Day 42 post LVS vaccination
Rabbit #66
Day 42 post LVS vaccination

FIG. 14A


Rabbit #36
Day -7
Rabbit #36
Day 42 post LVS vaccination
(Scarification)
Rabbit #43
Day -7
Rabbit #43
Day 42 post LVS vaccination
(Scarification)
Rabbit #48
Day -7
Rabbit #48
Day 42 post LVS vaccination
(Scarification)

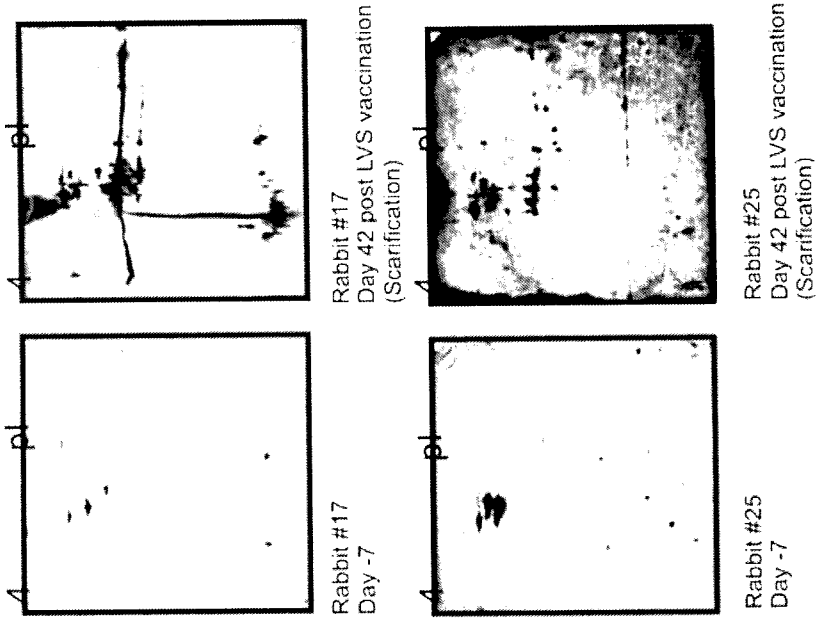

Rabbit #17
Day -7
Rabbit #17
Day 42 post LVS vaccination
(Scarification)
Rabbit #25
Day -7
Rabbit #25
Day 42 post LVS vaccination
(Scarification)

FIG. 14B

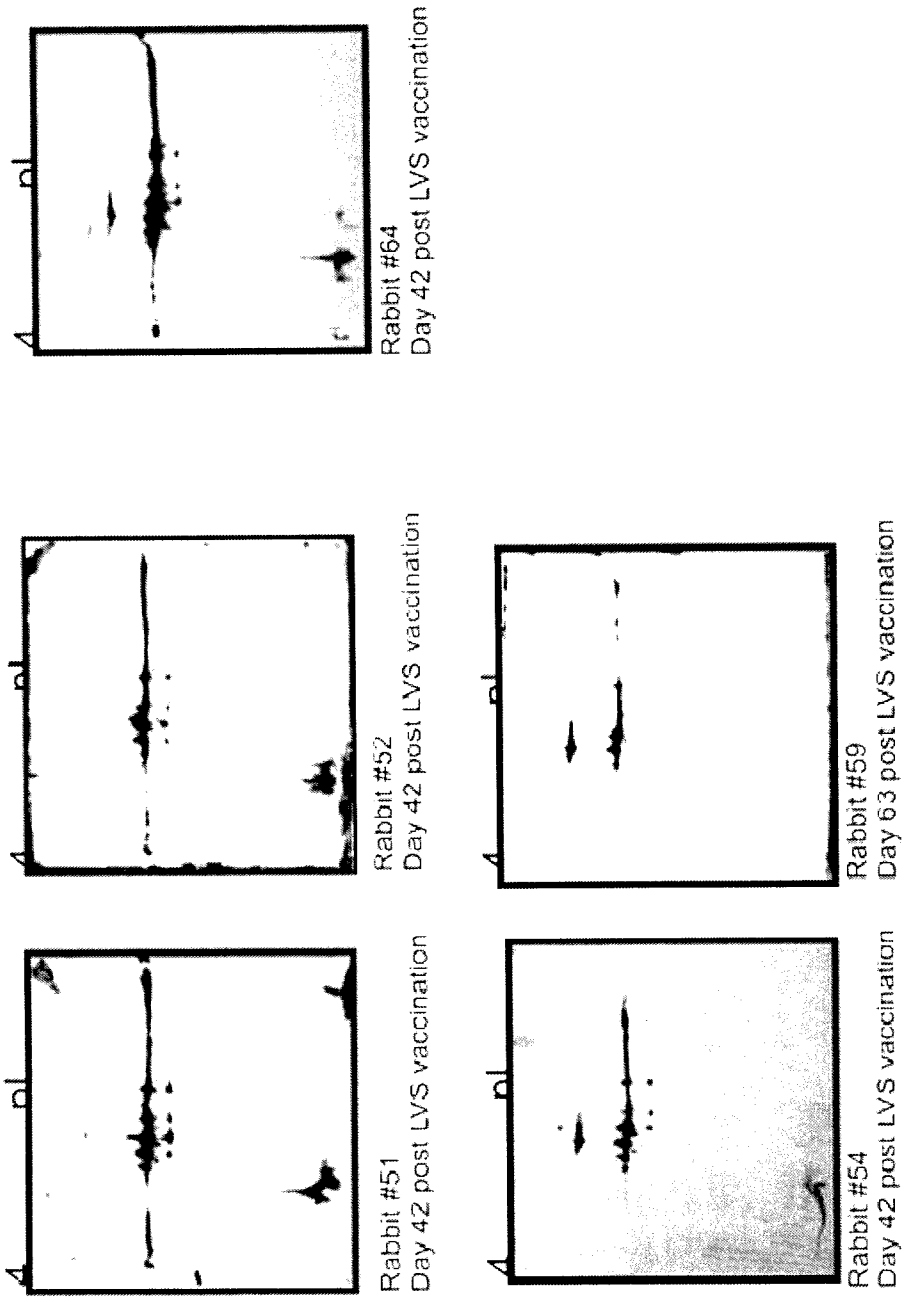

pl
Rabbit #51
Day 42 post LVS vaccination
pl
Rabbit #52
Day 42 post LVS vaccination
pl
Rabbit #64
Day 42 post LVS vaccination
pl
Rabbit #54
Day 42 post LVS vaccination
pl
Rabbit #59
Day 63 post LVS vaccination

FIG. 14B

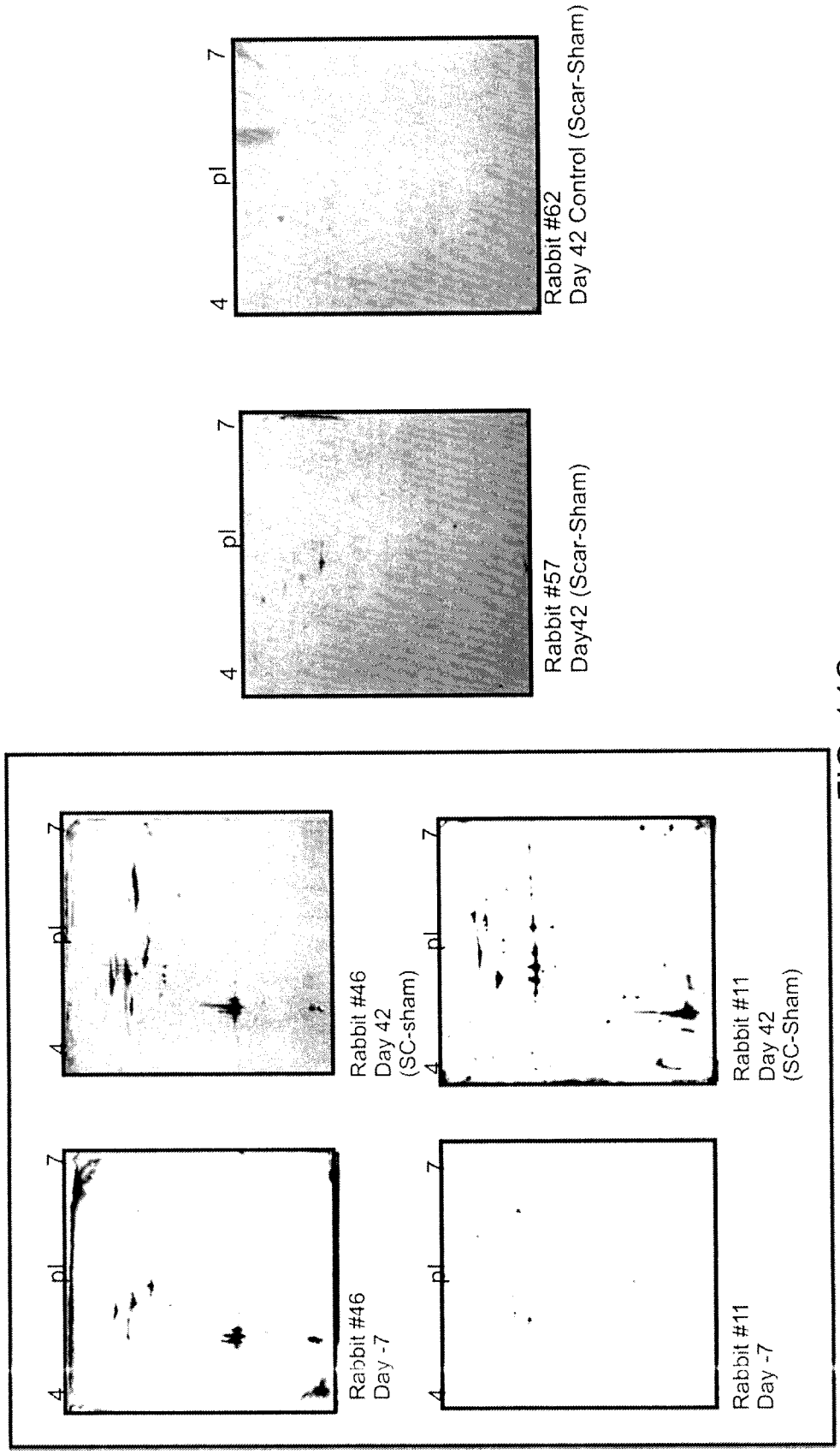

4
pI
7
Rabbit #46
Day -7
Rabbit #46
Day 42
(SC-sham)
Rabbit #11
Day -7
Rabbit #11
Day 42
(SC-Sham)
Rabbit #57
Day42 (Scar-Sham)
Rabbit #62
Day 42 Control (Scar-Sham)

FIG. 14C

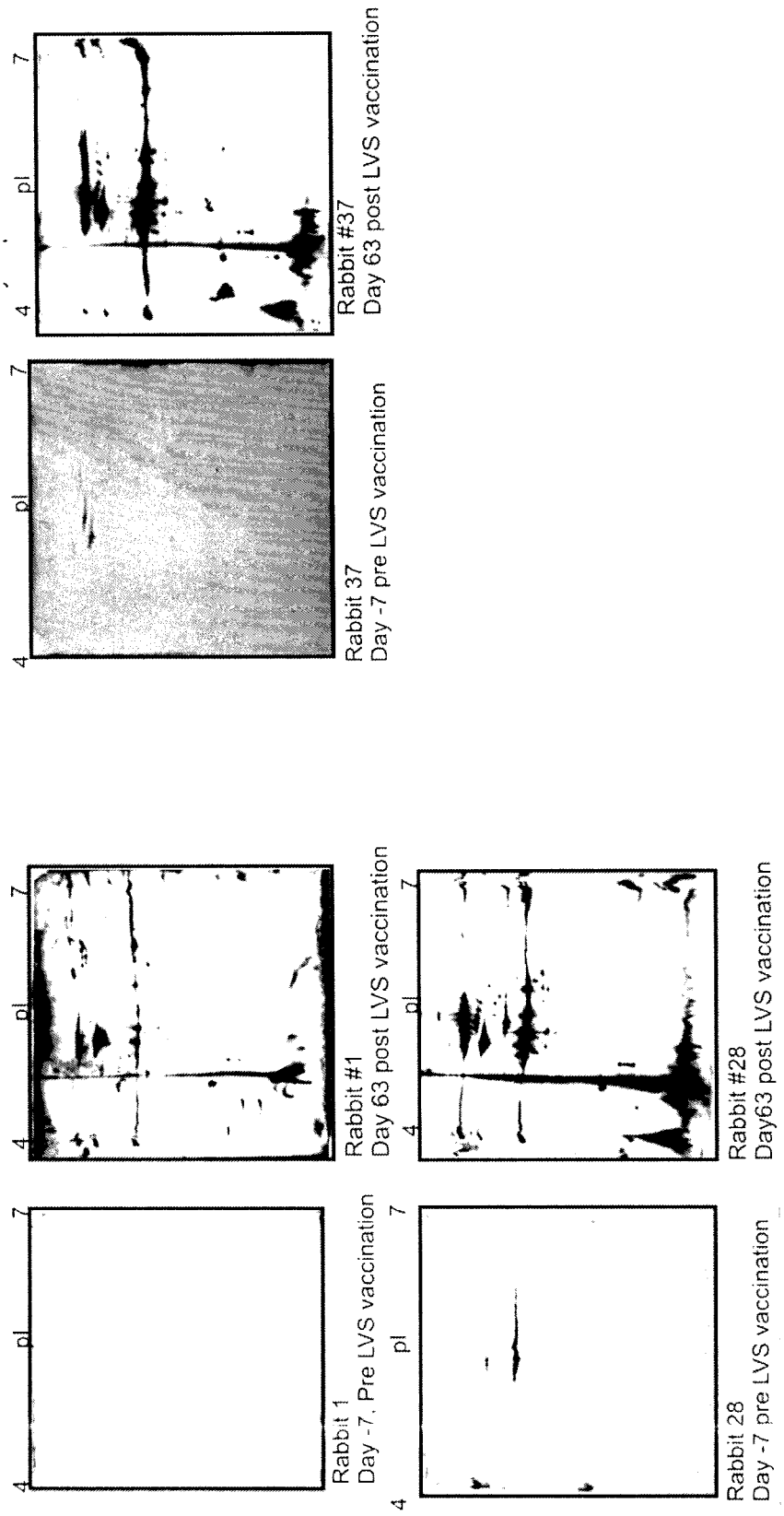

Rabbit #37
Day 63 post LVS vaccination
Rabbit 37
Day -7 pre LVS vaccination
Rabbit #1
Day 63 post LVS vaccination
Rabbit #28
Day63 post LVS vaccination
Rabbit 1
Day -7, Pre LVS vaccination
Rabbit 28
Day -7 pre LVS vaccination
pI
4
7

FIG. 14D

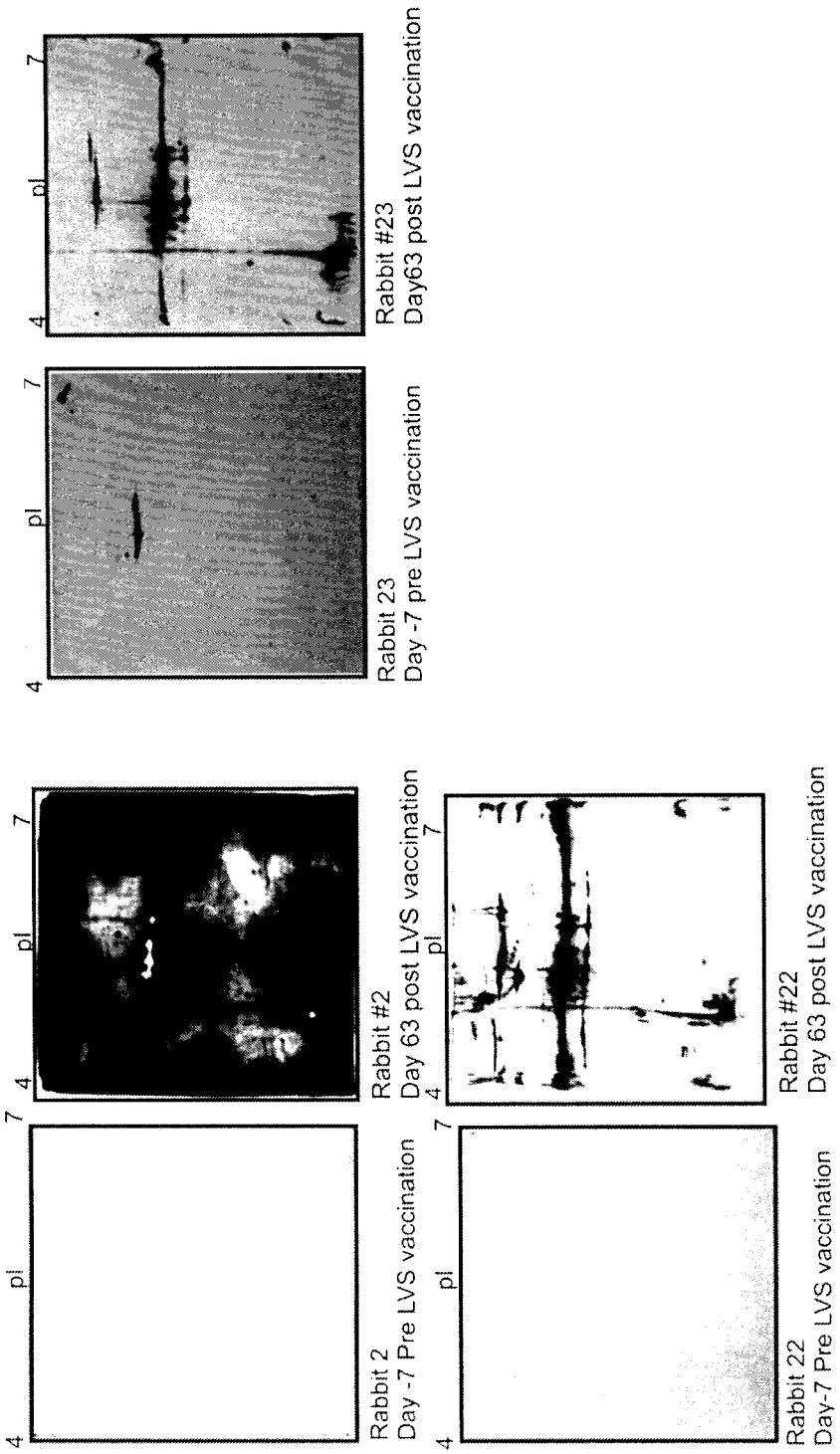
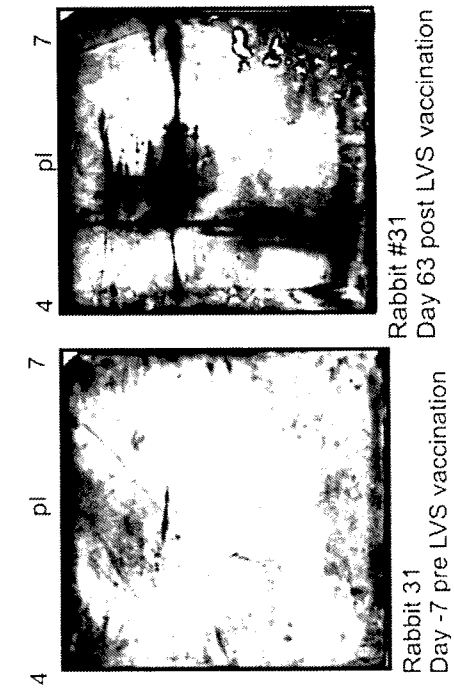

Rabbit 2
Day -7 Pre LVS vaccination
Rabbit #2
Day 63 post LVS vaccination
Rabbit 22
Day-7 Pre LVS vaccination
Rabbit #22
Day 63 post LVS vaccination
Rabbit 23
Day -7 pre LVS vaccination
Rabbit #23
Day63 post LVS vaccination
pi
4
7

FIG. 14D


4
pl
7
Rabbit 31
Day -7 pre LVS vaccination
Rabbit #31
Day 63 post LVS vaccination

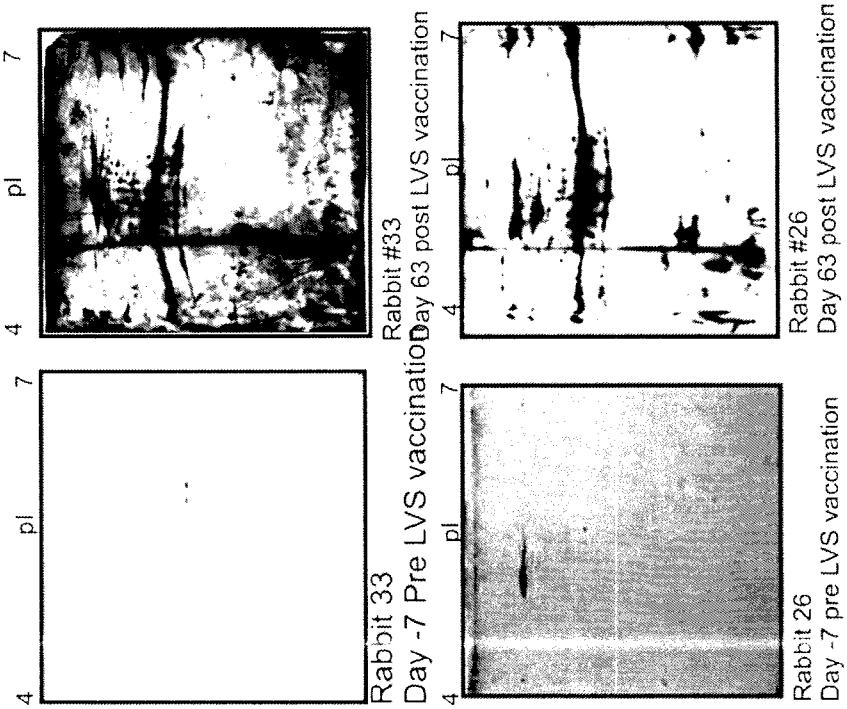

4
pl
7
Rabbit 33
Day -7 Pre LVS vaccination
Rabbit #33
Day 63 post LVS vaccination
Rabbit 26
Day -7 pre LVS vaccination
Rabbit #26
Day 63 post LVS vaccination

FIG. 14D

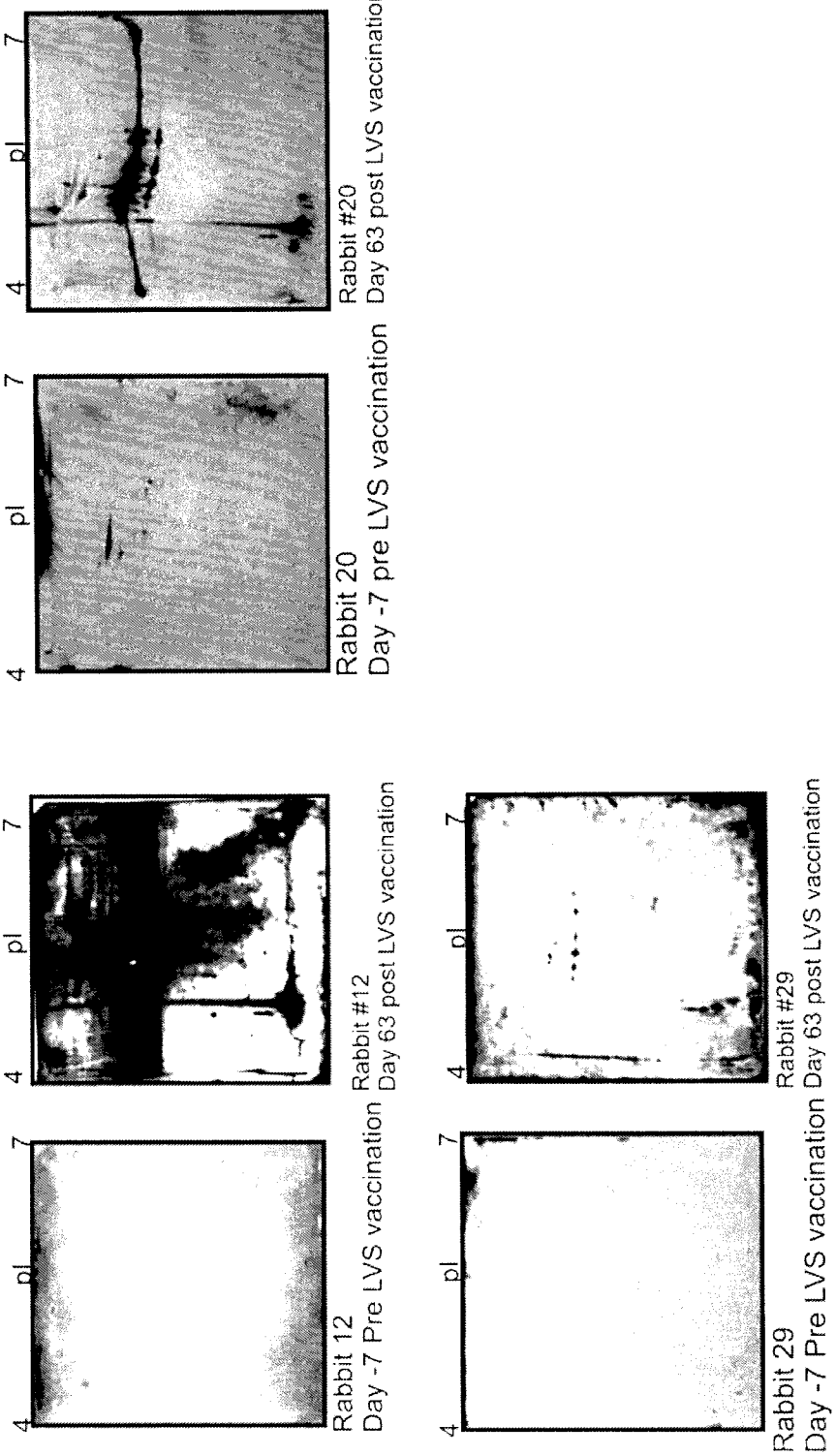

4
pl
7
Rabbit 12
Day -7 Pre LVS vaccination
Rabbit 12
Day 63 post LVS vaccination
Rabbit 20
Day -7 pre LVS vaccination
Rabbit #20
Day 63 post LVS vaccination
Rabbit 29
Day -7 Pre LVS vaccination
Rabbit #29
Day 63 post LVS vaccination

FIG. 14E

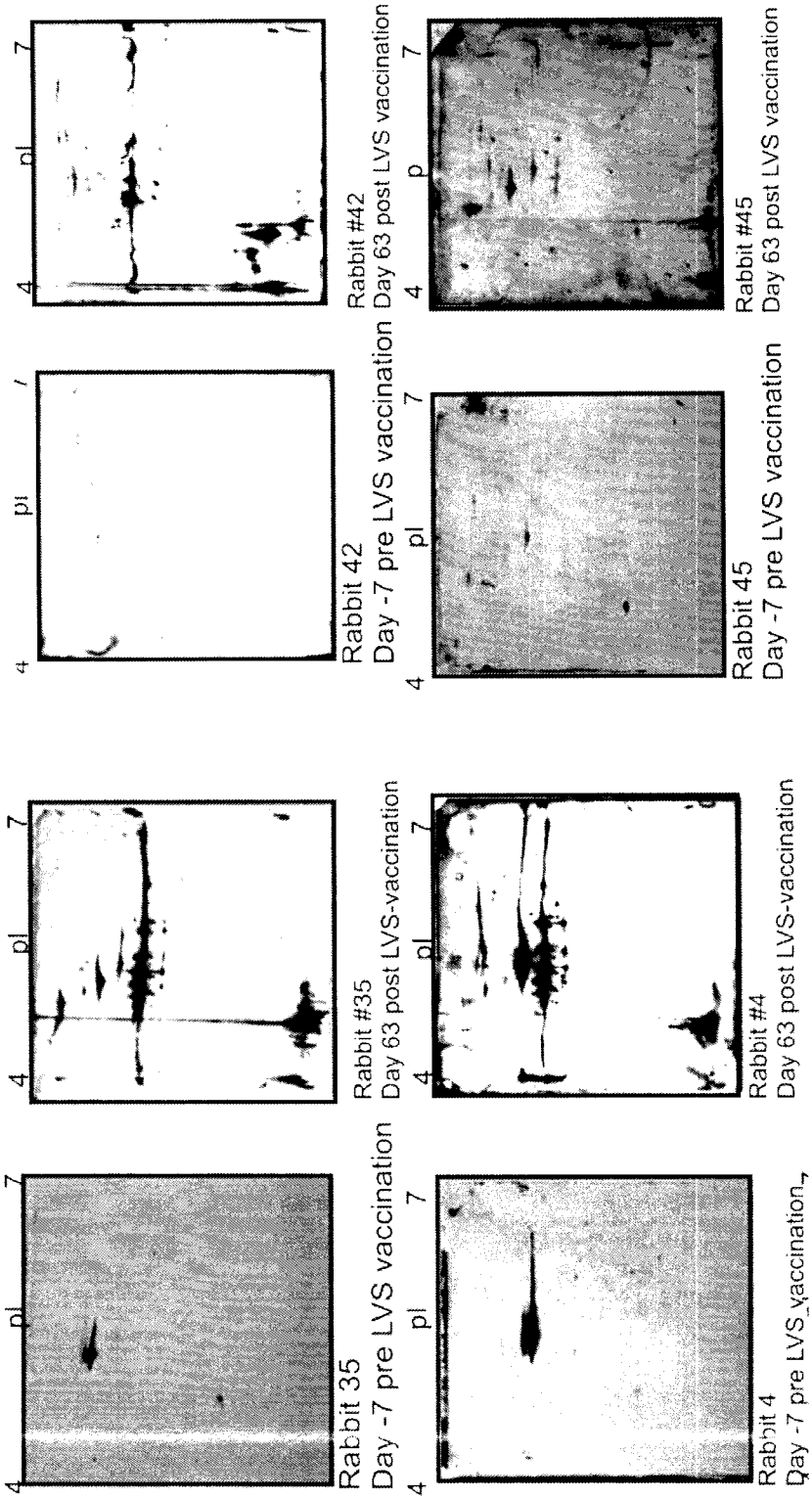

Rabbit 35
Day -7 pre LVS vaccination
Rabbit 4
Day -7 pre LVS vaccination
Rabbit 42
Day -7 pre LVS vaccination
Rabbit 45
Day -7 pre LVS vaccination
Rabbit #35
Day 63 post LVS-vaccination
Rabbit #4
Day 63 post LVS-vaccination
Rabbit #42
Day 63 post LVS vaccination
Rabbit #45
Day 63 post LVS vaccination
pI
4
7

FIG. 14E

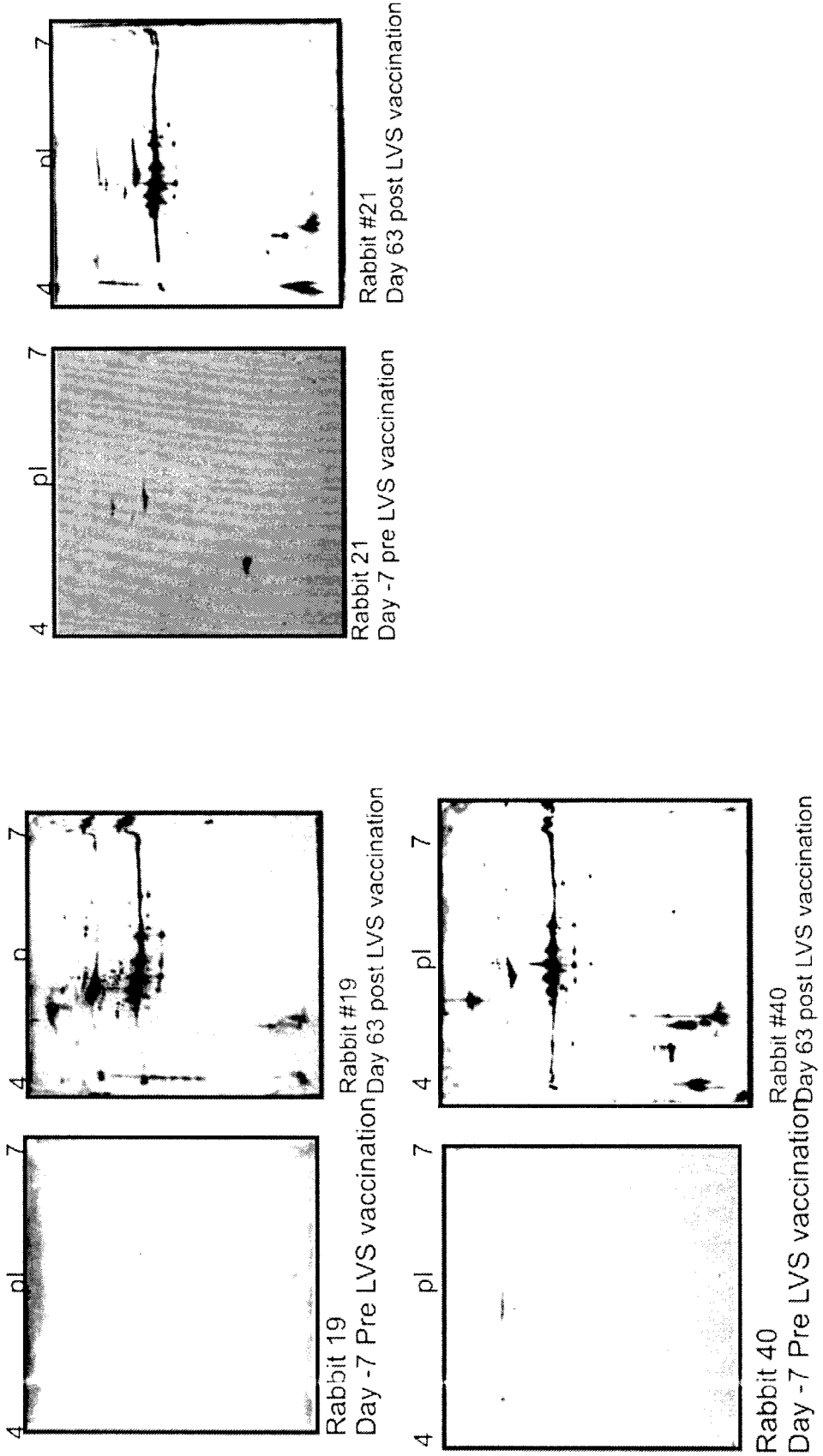


FIG. 14E (CONT'D)

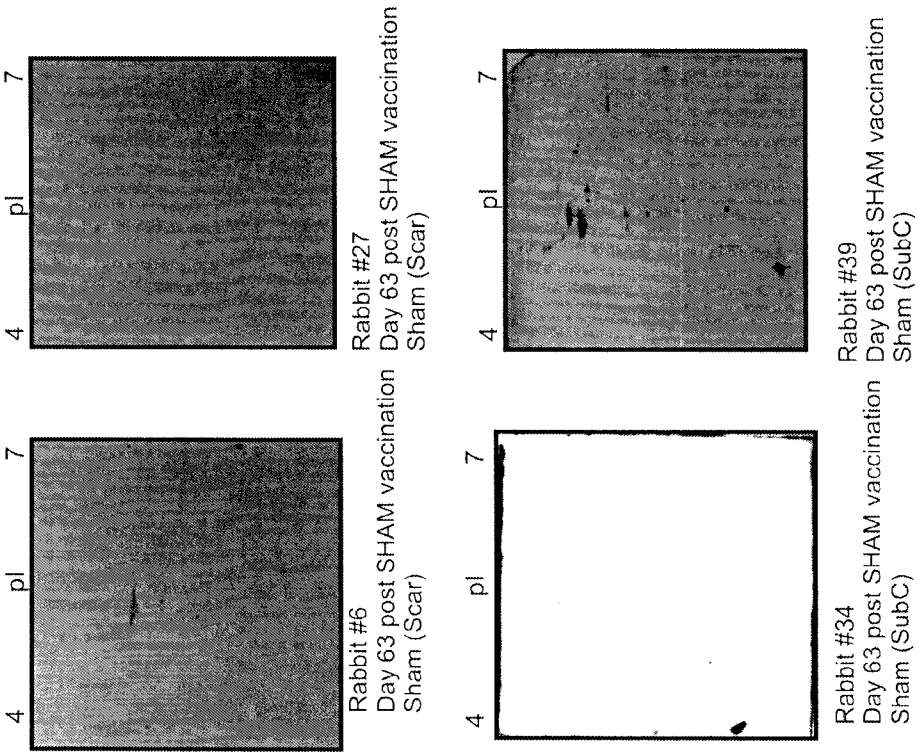

4
pl
7
Rabbit #6
Day 63 post SHAM vaccination
Sham (Scar)
Rabbit #27
Day 63 post SHAM vaccination
Sham (Scar)
Rabbit #34
Day 63 post SHAM vaccination
Sham (SubC)
Rabbit #39
Day 63 post SHAM vaccination
Sham (SubC)

FIG. 14F

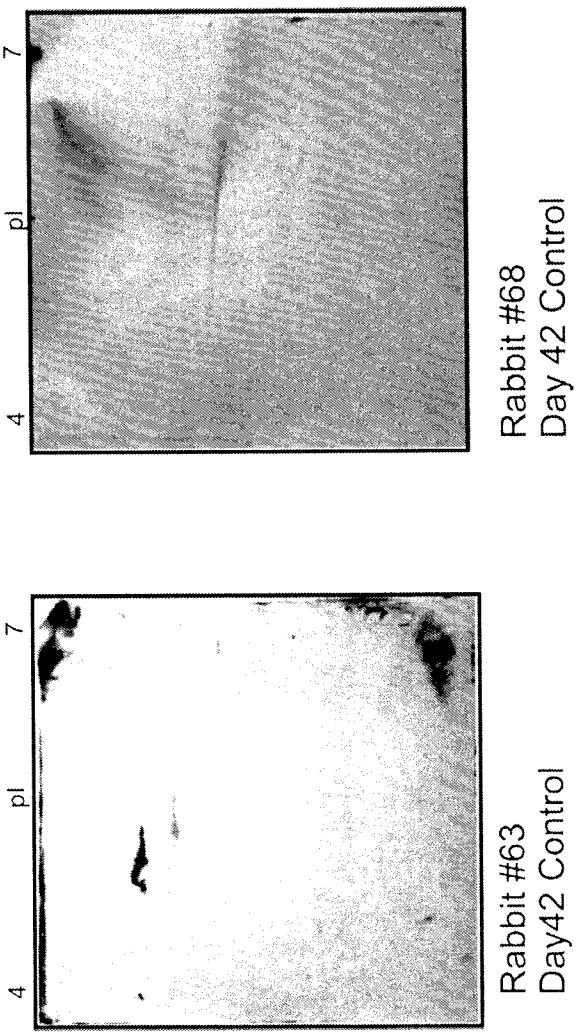
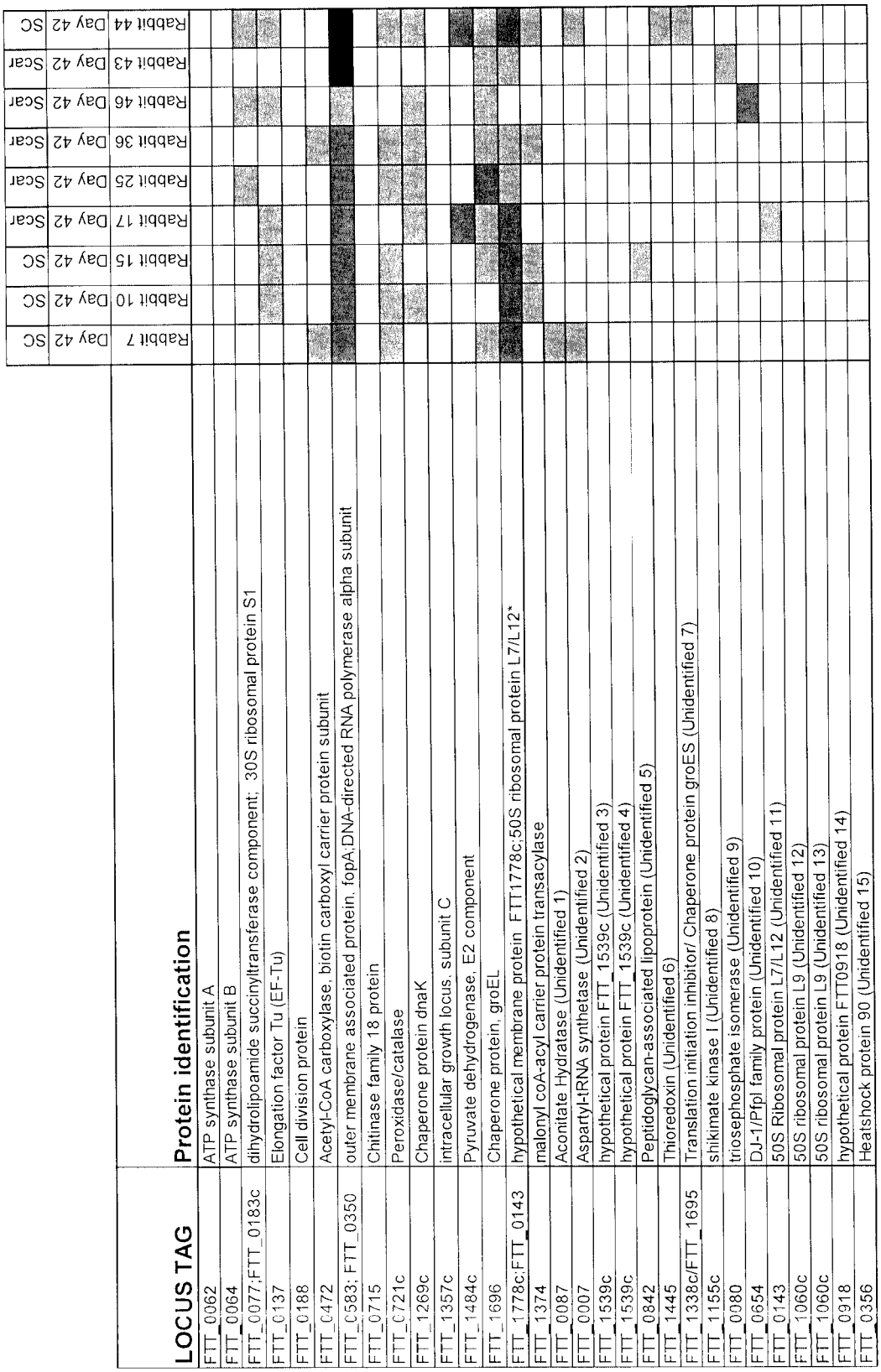


FIG. 14G



LOCUS TAG	Protein identification	Rabbit 7 Day 42 SC	Rabbit 10 Day 42 SC	Rabbit 15 Day 42 SC	Rabbit 17 Day 42 Scar	Rabbit 25 Day 42 Scar	Rabbit 36 Day 42 Scar	Rabbit 46 Day 42 Scar	Rabbit 43 Day 42 Scar	Rabbit 44 Day 42 SC
FTT_0062	ATP synthase subunit A									
FTT_0064	ATP synthase subunit B									
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase component; 30S ribosomal protein S1									
FTT_0137	Elongation factor Tu (EF-Tu)									
FTT_0188	Cell division protein									
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit									
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit									
FTT_0715	Chitinase family 18 protein									
FTT_0721c	Peroxidase/catalase									
FTT_1269c	Chaperone protein dnaK									
FTT_1357c	intracellular growth locus, subunit C									
FTT_1484c	Pyruvate dehydrogenase, E2 component									
FTT_1696	Chaperone protein, groEL									
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*									
FTT_1374	malonyl coA-acyl carrier protein transacylase									
FTT_0087	Aconitate Hydratase (Unidentified 1)									
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)									
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)									
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)									
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)									
FTT_1445	Thioredoxin (Unidentified 6)									
FTT_1338c/FTT_1695	Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)									
FTT_1155c	shikimate kinase I (Unidentified 8)									
FTT_0080	triosephosphate isomerase (Unidentified 9)									
FTT_0654	DJ-1/Pfpl family protein (Unidentified 10)									
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)									
FTT_1060c	50S ribosomal protein L9 (Unidentified 12)									
FTT_1060c	50S ribosomal protein L9 (Unidentified 13)									
FTT_0918	hypothetical protein FTT0918 (Unidentified 14)									
FTT_0356	Heatshock protein 90 (Unidentified 15)									

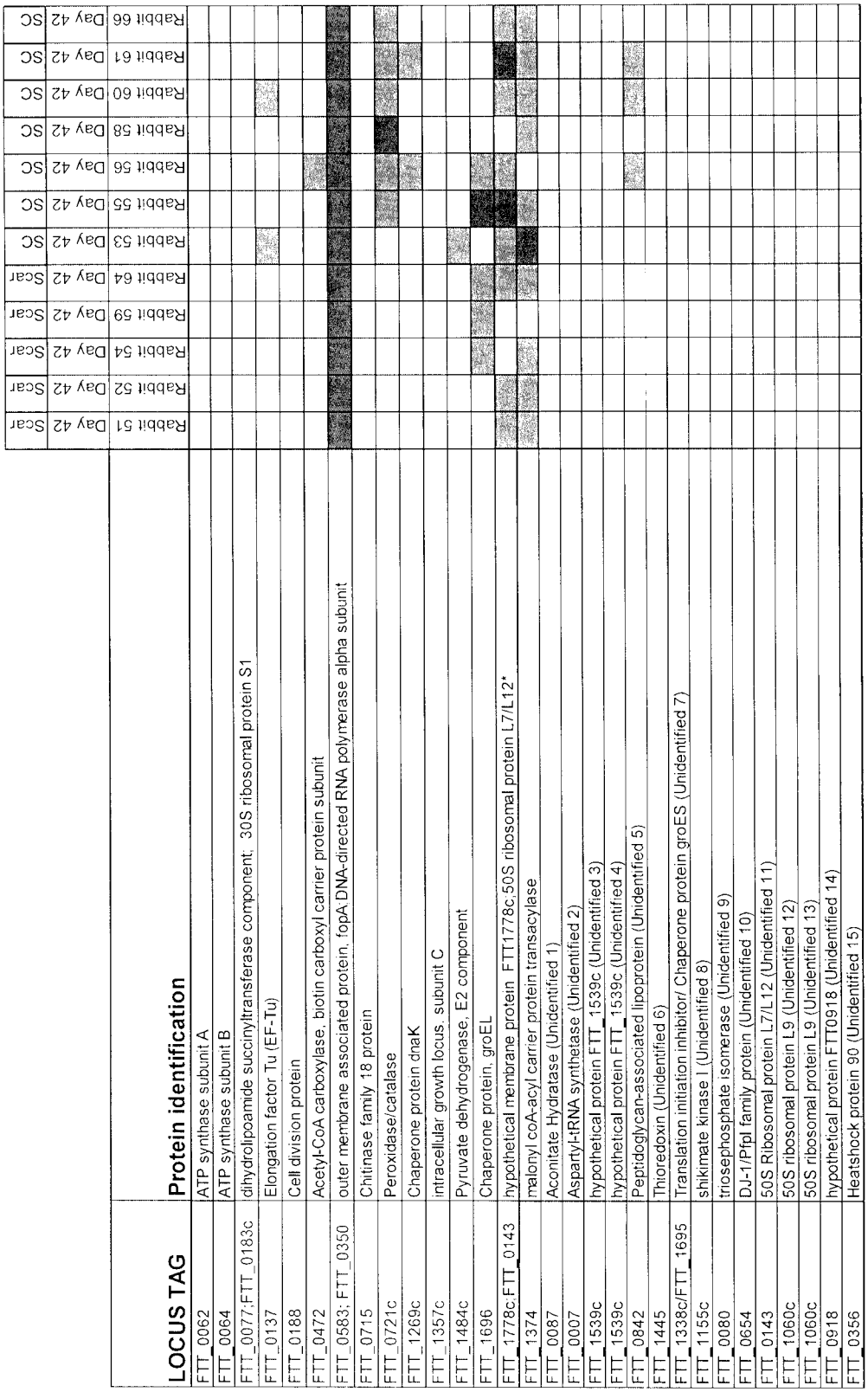
FIG. 15

LOCUS TAG	Protein identification	Rabbit 1	Day 63	SC	Rabbit 2	Day63	SC	Rabbit 22	Day63	SC	Rabbit 23	Day63	SC	Rabbit 26	Day63	SC	Rabbit 28	Day63	SC	Rabbit 31	Day63	SC	Rabbit 33	Day63	SC	Rabbit 37	Day63	SC
FTT_0002	ATP synthase subunit A																											
FTT_0004	ATP synthase subunit B																											
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase component; 30S ribosomal protein S1																											
FTT_0137	Elongation factor Tu (EF-Tu)																											
FTT_0188	Cell division protein																											
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit																											
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit																											
FTT_0715	Chitinase family 18 protein																											
FTT_0721c	Peroxidase/catalase																											
FTT_1269c	Chaperone protein dnaK																											
FTT_1357c	intracellular growth locus, subunit C																											
FTT_1484c	Pyruvate dehydrogenase, E2 component																											
FTT_1696	Chaperone protein, groEL																											
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*																											
FTT_1374	malonyl coA-acyl carrier protein transacylase																											
FTT_0037	Aconitate Hydratase (Unidentified 1)																											
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)																											
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)																											
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)																											
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)																											
FTT_1445	Thioredoxin (Unidentified 6)																											
FTT_1338c/FTT_1695	Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)																											
FTT_1155c	shikimate kinase I (Unidentified 8)																											
FTT_0080	triosephosphate isomerase (Unidentified 9)																											
FTT_0654	DJ-1/Pfpl family protein (Unidentified 10)																											
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)																											
FTT_1060c	50S ribosomal protein L9 (Unidentified 12)																											
FTT_1060c	50S ribosomal protein L9 (Unidentified 13)																											
FTT_0918	hypothetical protein FTT0918 (Unidentified 14)																											
FTT_0356	Heatshock protein 90 (Unidentified 15)																											

FIG. 15

LOCUS TAG	Protein identification	Rabbit 4 Day63 Scar	Rabbit 12 Day63 Scar	Rabbit 19 Day63 Scar	Rabbit 20 Day63 Scar	Rabbit 21 Day63 Scar	Rabbit 29 Day63 Scar	Rabbit 35 Day63 Scar	Rabbit 40 Day63 Scar	Rabbit 42 Day63 Scar	Rabbit 45 Day63 Scar
FTT_0062	ATP synthase subunit A										
FTT_0064	ATP synthase subunit B										
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase component; 30S ribosomal protein S1										
FTT_0137	Elongation factor Tu (EF-Tu)										
FTT_0188	Cell division protein										
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit										
FTT_0583; FTT_0350	outer membrane associated protein, fopA;DNA-directed RNA polymerase alpha subunit										
FTT_0715	Chitinase family 18 protein										
FTT_0721c	Peroxidase/catalase										
FTT_1269c	Chaperone protein dnaK										
FTT_1357c	intracellular growth locus, subunit C										
FTT_1484c	Pyruvate dehydrogenase, E2 component										
FTT_1696	Chaperone protein, groEL										
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*										
FTT_1374	malonyl coA-acyl carrier protein transacylase										
FTT_0087	Aconitate Hydratase (Unidentified 1)										
FTT_0007	AspartyI-tRNA synthetase (Unidentified 2)										
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)										
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)										
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)										
FTT_1445	Thioredoxin (Unidentified 6)										
FTT_1338c/FTT_1695	Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)										
FTT_1155c	shikimate kinase I (Unidentified 8)										
FTT_0080	triosephosphate isomerase (Unidentified 9)										
FTT_0654	DJ-1/PfpI family protein (Unidentified 10)										
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)										
FTT_1060c	50S ribosomal protein L9 (Unidentified 12)										
FTT_1060c	50S ribosomal protein L9 (Unidentified 13)										
FTT_0918	hypothetical protein FTT0918 (Unidentified 14)										
FTT_0356	Heatshock protein 90 (Unidentified 15)										

FIG. 15



LOCUS TAG	Protein identification	Rabbit 51 Day 42 Scar	Rabbit 52 Day 42 Scar	Rabbit 54 Day 42 Scar	Rabbit 59 Day 42 Scar	Rabbit 64 Day 42 Scar	Rabbit 53 Day 42 SC	Rabbit 55 Day 42 SC	Rabbit 56 Day 42 SC	Rabbit 58 Day 42 SC	Rabbit 60 Day 42 SC	Rabbit 61 Day 42 SC	Rabbit 66 Day 42 SC
FTT_0062	ATP synthase subunit A												
FTT_0064	ATP synthase subunit B												
FTT_0077;FTT_0183c	dihydrolipoamide succinyltransferase component; 30S ribosomal protein S1												
FTT_0137	Elongation factor Tu (EF-Tu)												
FTT_0188	Cell division protein												
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit												
FTT_0583; FTT_0350	outer membrane associated protein, fopA DNA-directed RNA polymerase alpha subunit												
FTT_0715	Chitinase family 18 protein												
FTT_0721c	Peroxidase/catalase												
FTT_1269c	Chaperone protein dnaK												
FTT_1357c	intracellular growth locus, subunit C												
FTT_1484c	Pyruvate dehydrogenase, E2 component												
FTT_1696	Chaperone protein, groEL												
FTT_1778c;FTT_0143	hypothetical membrane protein FTT1778c;50S ribosomal protein L7/L12*												
FTT_1374	malonyl coA-acyl carrier protein transacylase												
FTT_0087	Aconitate Hydratase (Unidentified 1)												
FTT_0007	Aspartyl-tRNA synthetase (Unidentified 2)												
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 3)												
FTT_1539c	hypothetical protein FTT_1539c (Unidentified 4)												
FTT_0842	Peptidoglycan-associated lipoprotein (Unidentified 5)												
FTT_1445	Thioredoxin (Unidentified 6)												
FTT_1338c/FTT_1695	Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)												
FTT_1155c	shikimate kinase I (Unidentified 8)												
FTT_0080	triosephosphate isomerase (Unidentified 9)												
FTT_0654	DJ-1/PfpI family protein (Unidentified 10)												
FTT_0143	50S Ribosomal protein L7/L12 (Unidentified 11)												
FTT_1060c	50S ribosomal protein L9 (Unidentified 12)												
FTT_1060c	50S ribosomal protein L9 (Unidentified 13)												
FTT_0918	hypothetical protein FTT0918 (Unidentified 14)												
FTT_0356	Heatshock protein 90 (Unidentified 15)												

FIG. 15

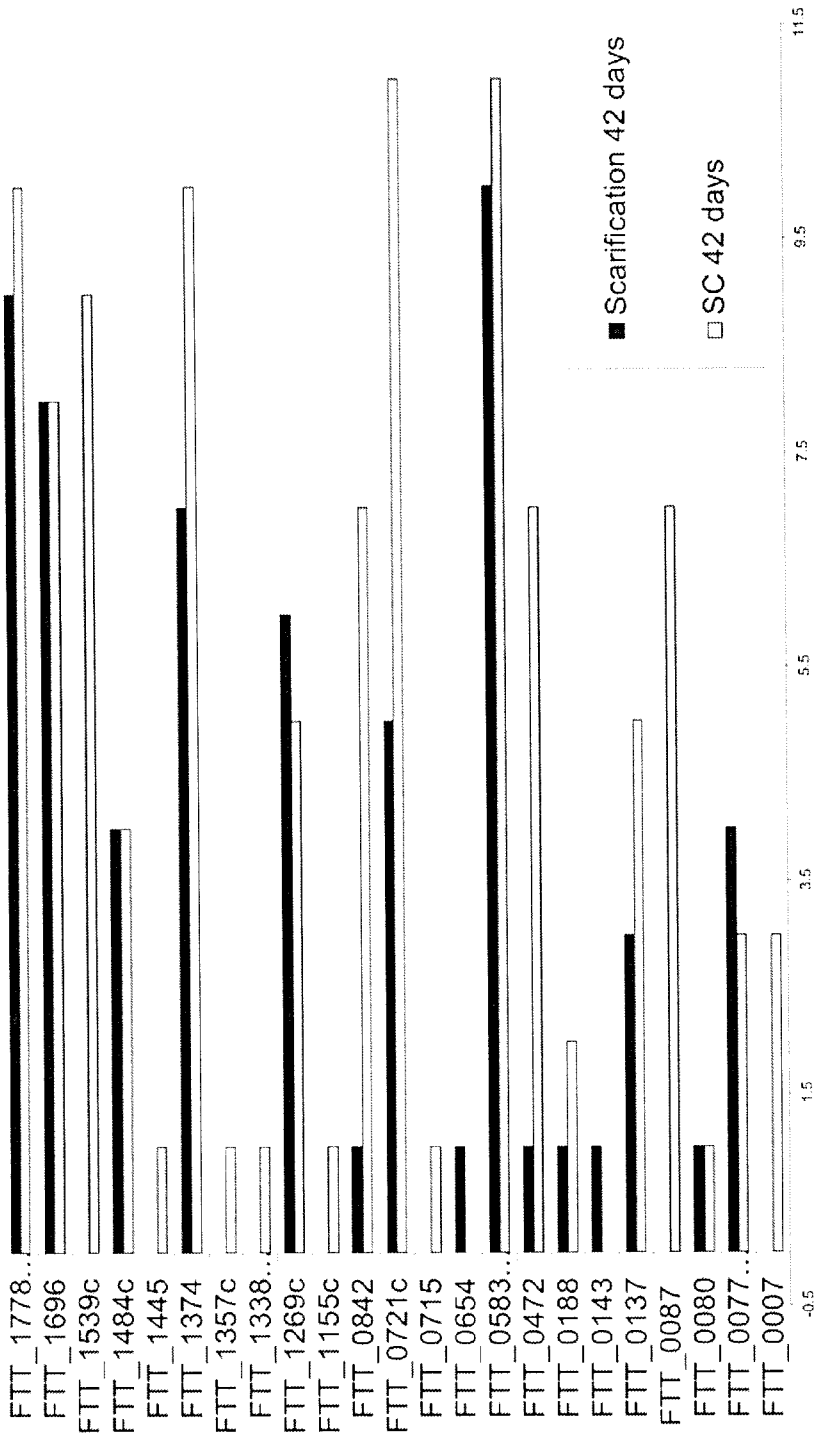

FTT_1778...
FTT_1696
FTT_1539c
FTT_1484c
FTT_1445
FTT_1374
FTT_1357c
FTT_1338...
FTT_1269c
FTT_1155c
FTT_0842
FTT_0721c
FTT_0715
FTT_0654
FTT_0583...
FTT_0472
FTT_0188
FTT_0143
FTT_0137
FTT_0087
FTT_0080
FTT_0077...
FTT_0007
-0.5
1.5
3.5
5.5
7.5
9.5
11.5
Scarification 42 days
SC 42 days
Relative observed intensity of immunoreactivity

FIG. 16A

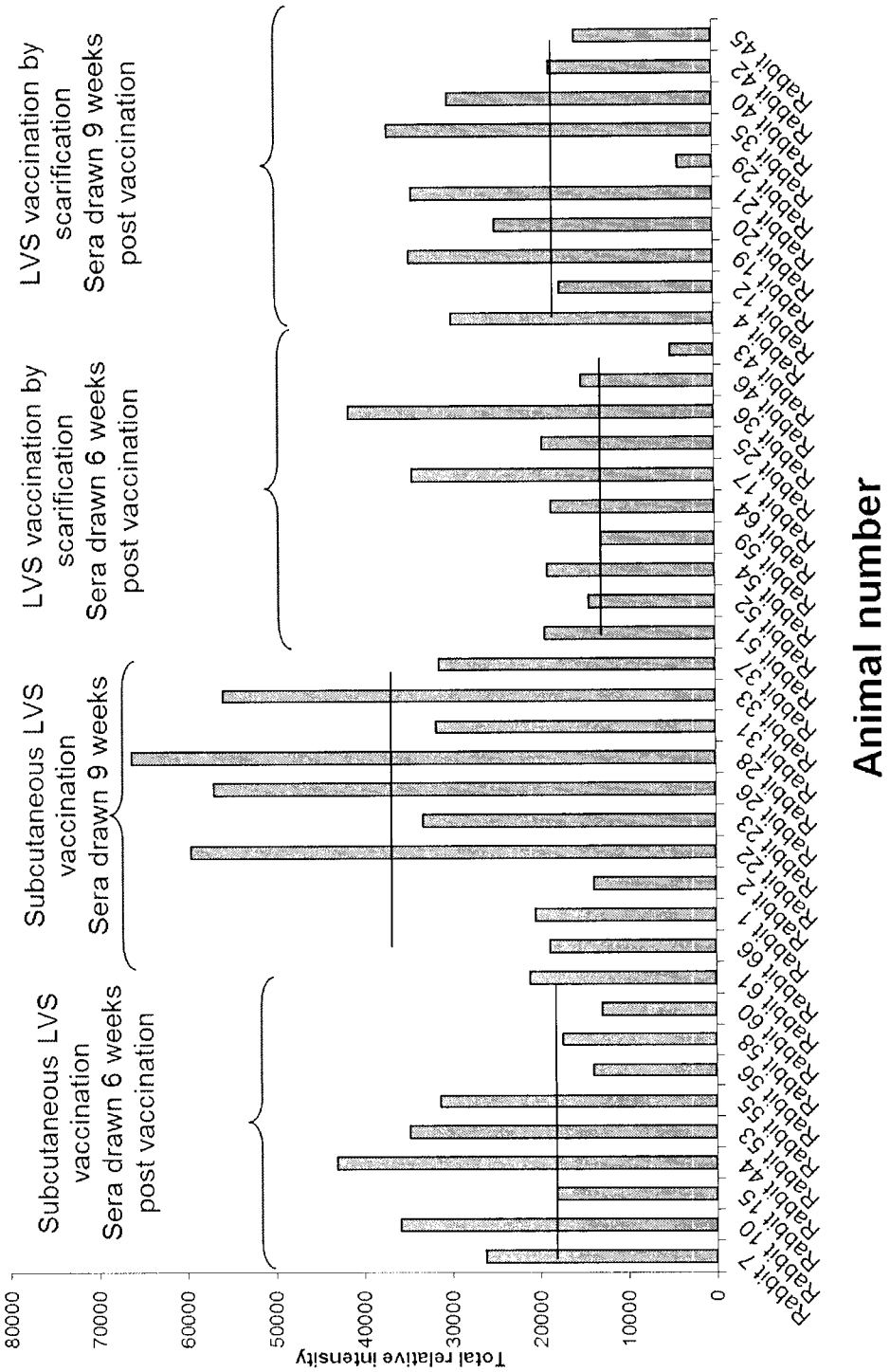

Subcutaneous LVS vaccination Sera drawn 6 weeks post vaccination
Subcutaneous LVS vaccination Sera drawn 9 weeks
LVS vaccination by scarification Sera drawn 6 weeks post vaccination
LVS vaccination by scarification Sera drawn 9 weeks post vaccination
Total relative intensity
0
10000
20000
30000
40000
50000
60000
70000
80000
Animal number
Rabbit 7
Rabbit 10
Rabbit 15
Rabbit 44
Rabbit 53
Rabbit 55
Rabbit 56
Rabbit 58
Rabbit 60
Rabbit 61
Rabbit 66
Rabbit 1
Rabbit 22
Rabbit 23
Rabbit 26
Rabbit 28
Rabbit 31
Rabbit 33
Rabbit 37
Rabbit 51
Rabbit 52
Rabbit 54
Rabbit 59
Rabbit 64
Rabbit 17
Rabbit 25
Rabbit 36
Rabbit 46
Rabbit 43
Rabbit 4
Rabbit 12
Rabbit 19
Rabbit 20
Rabbit 21
Rabbit 29
Rabbit 35
Rabbit 40
Rabbit 42
Rabbit 45

FIG. 16B

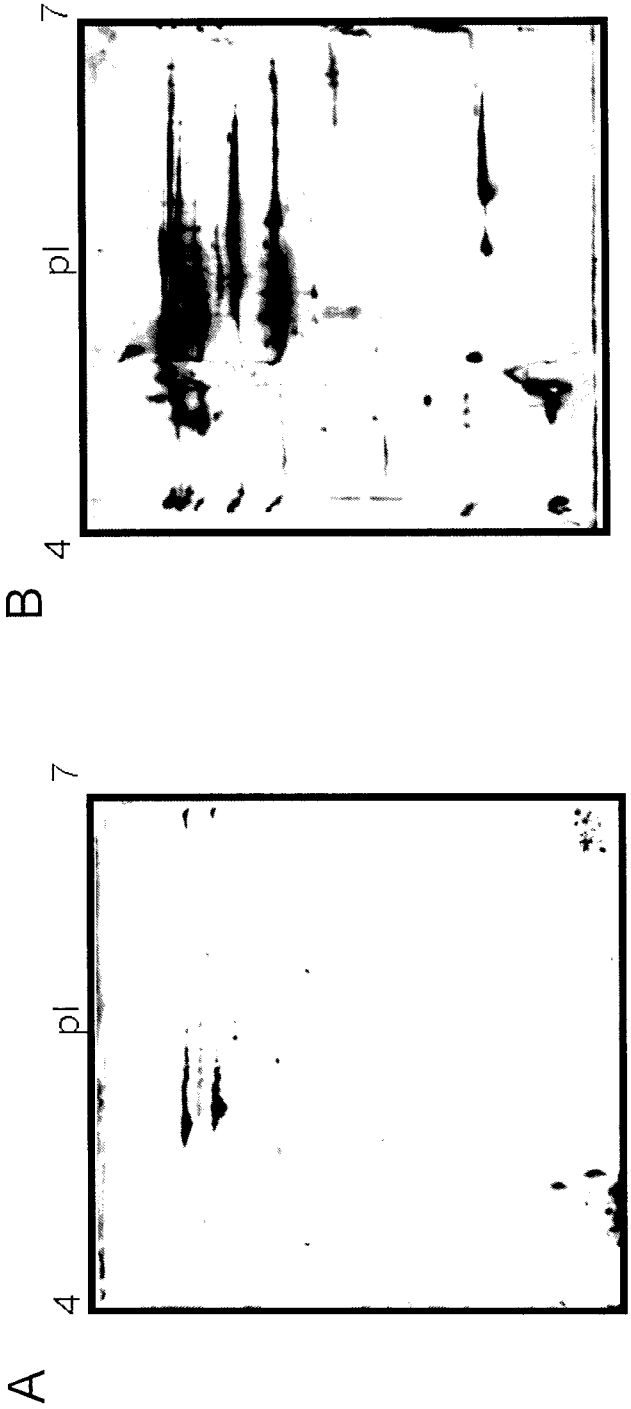


FIG. 17

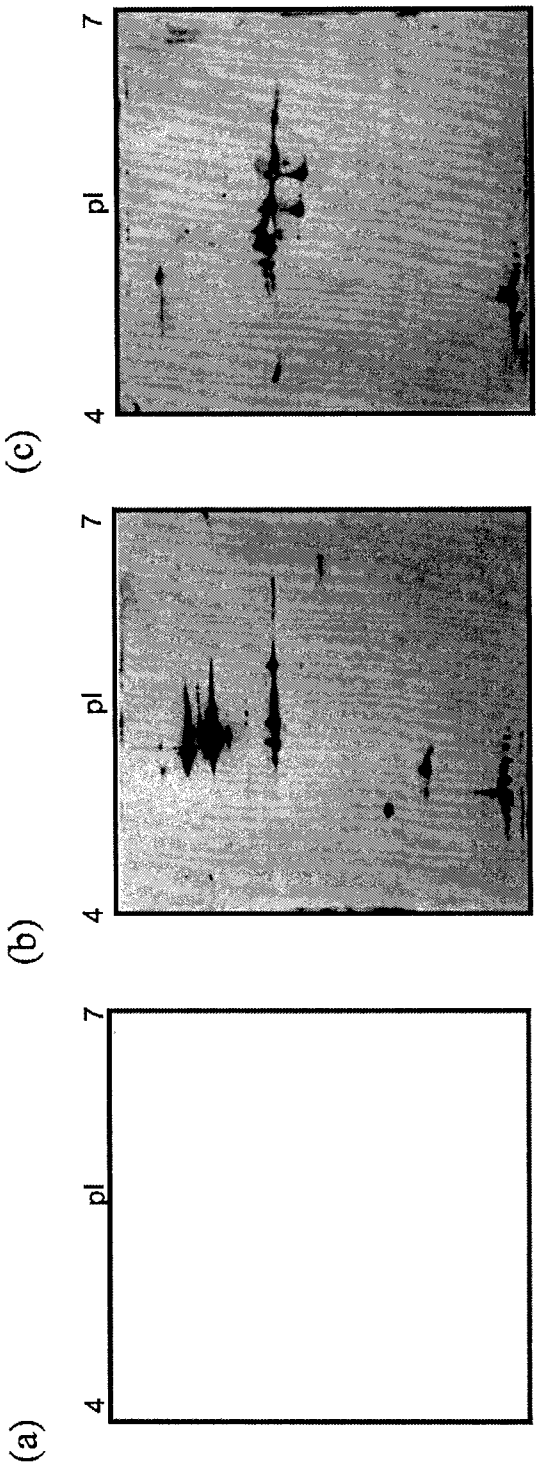


FIG. 18

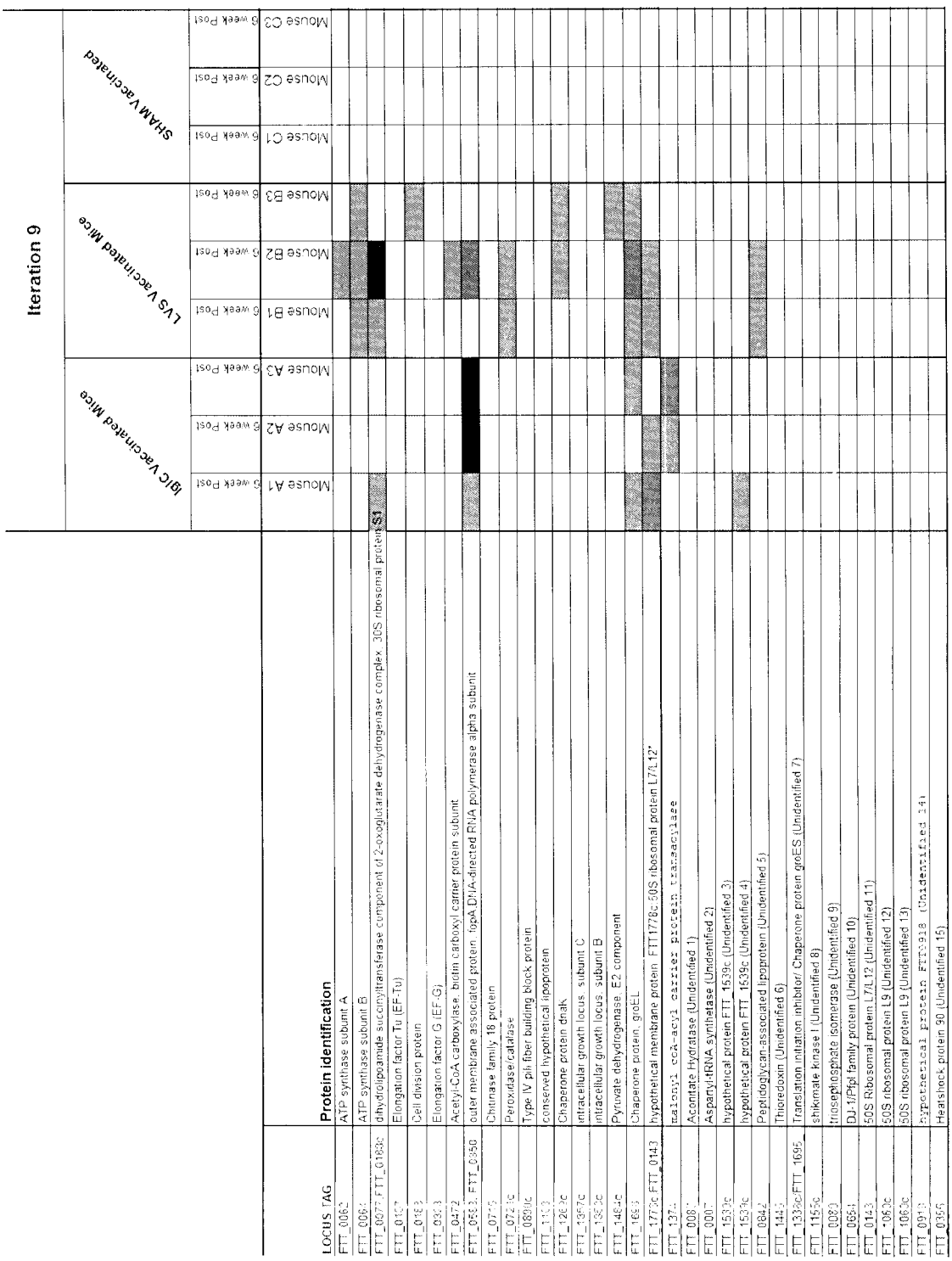

Iteration 9
IglC Vaccinated Mice
LVS Vaccinated Mice
SHAM Vaccinated
LOCUS TAG
Protein identification
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C1
Mouse C2
Mouse C3
6 week Post
FTT_0082
ATP synthase subunit A
FTT_0084
ATP synthase subunit B
FTT_0087 FTT_0183c
dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex
30S ribosomal protein S1
FTT_0157
Elongation factor Tu (EF-Tu)
FTT_0183
Cell division protein
FTT_0303
Elongation factor G (EF-G)
FTT_0472
Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit
FTT_0563, FTT_0950
outer membrane associated protein, topA DNA-directed RNA polymerase alpha subunit
FTT_0775
Chitinase family 18 protein
FTT_0782c
Peroxidase/catalase
FTT_0890c
Type IV pili fiber building block protein
FTT_1109
conserved hypothetical lipoprotein
FTT_1263c
Chaperone protein dnaK
FTT_1367c
intracellular growth locus, subunit C
FTT_1383c
intracellular growth locus, subunit B
FTT_1464c
Pyruvate dehydrogenase, E2 component
FTT_1693
Chaperone protein, groEL
FTT_1778c FTT_0143
hypothetical membrane protein
FTT1778c 50S ribosomal protein L7/L12"
FTT_1378
malonyl coA-acyl carrier protein transacylase
FTT_0081
Aconitate Hydratase (Unidentified 1)
FTT_0007
Aspartyl-tRNA synthetase (Unidentified 2)
FTT_1539c
hypothetical protein FTT_1539c (Unidentified 3)
FTT_1539c
hypothetical protein FTT_1539c (Unidentified 4)
FTT_0842
Peptidoglycan-associated lipoprotein (Unidentified 5)
FTT_1443
Thioredoxin (Unidentified 6)
FTT_1336c FTT_1695
Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)
FTT_1155c
shikimate kinase I (Unidentified 8)
FTT_0080
triosephosphate isomerase (Unidentified 9)
FTT_0654
DJ-1/Pfpl family protein (Unidentified 10)
FTT_0143
50S Ribosomal protein L7/L12 (Unidentified 11)
FTT_1062c
50S ribosomal protein L9 (Unidentified 12)
FTT_1063c
50S ribosomal protein L9 (Unidentified 13)
FTT_0918
hypothetical protein FTT0918 (Unidentified 14)
FTT_0365
Heatshock protein 90 (Unidentified 15)

FIG. 19A

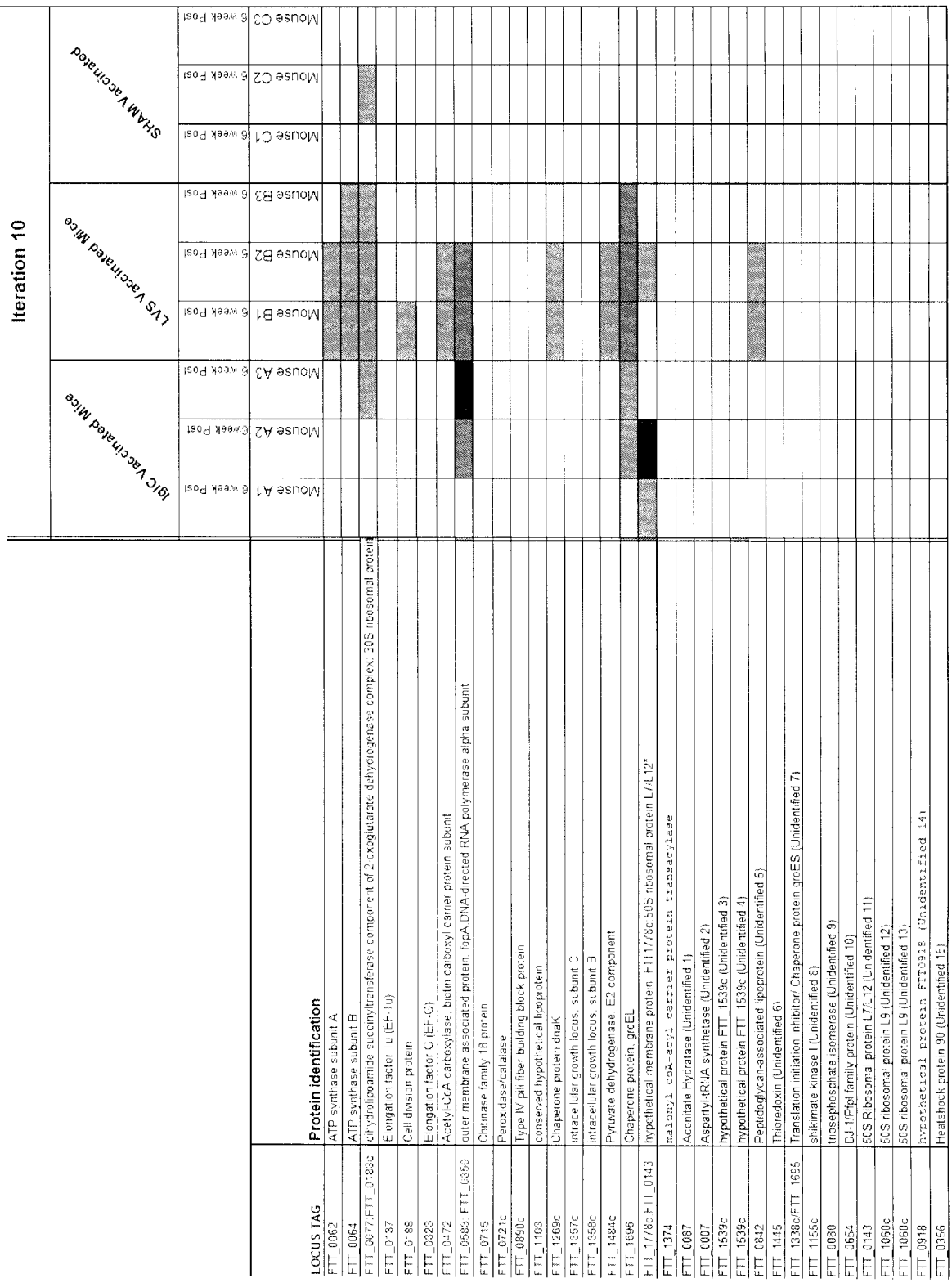

Iteration 10
IglC Vaccinated Mice
LVS Vaccinated Mice
SHAM Vaccinated
LOCUS TAG
Protein identification
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C1
Mouse C2
Mouse C3
6 week Post
FTT_0062 ATP synthase subunit A
FTT_0064 ATP synthase subunit B
FTT_0677/FTT_0783c dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, 30S ribosomal protein
FTT_0137 Elongation factor Tu (EF-Tu)
FTT_0188 Cell division protein
FTT_0323 Elongation factor G (EF-G)
FTT_0472 Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit
FTT_0583/FTT_0350 outer membrane associated protein, tolA DNA-directed RNA polymerase alpha subunit
FTT_0715 Chitinase family 18 protein
FTT_0721c Peroxidase/catalase
FTT_0890c Type IV pili fiber building block protein
FTT_1103 conserved hypothetical lipoprotein
FTT_1269c Chaperone protein dnaK
FTT_1357c intracellular growth locus, subunit C
FTT_1358c intracellular growth locus, subunit B
FTT_1484c Pyruvate dehydrogenase, E2 component
FTT_1696 Chaperone protein, groEL
FTT_1778c/FTT_0143 hypothetical membrane protein, FTT1778c 50S ribosomal protein L7/L12*
FTT_1374 malonyl coA-acyl carrier protein transacylase
FTT_0087 Aconitate Hydratase (Unidentified 1)
FTT_0007 Aspartyl-tRNA synthetase (Unidentified 2)
FTT_1539c hypothetical protein FTT_1539c (Unidentified 3)
FTT_1539c hypothetical protein FTT_1539c (Unidentified 4)
FTT_0842 Peptidoglycan-associated lipoprotein (Unidentified 5)
FTT_1445 Thioredoxin (Unidentified 6)
FTT_1338c/FTT_1695 Translation initiation inhibitor/ Chaperone protein groES (Unidentified 7)
FTT_1155c shikimate kinase I (Unidentified 8)
FTT_0080 triosephosphate isomerase (Unidentified 9)
FTT_0654 DJ-1/PfpI family protein (Unidentified 10)
FTT_0143 50S Ribosomal protein L7/L12 (Unidentified 11)
FTT_1060c 50S ribosomal protein L9 (Unidentified 12)
FTT_1060c 50S ribosomal protein L9 (Unidentified 13)
FTT_0918 hypothetical protein FTT0918 (Unidentified 14)
FTT_0356 Heatshock protein 90 (Unidentified 15)

FIG. 19A

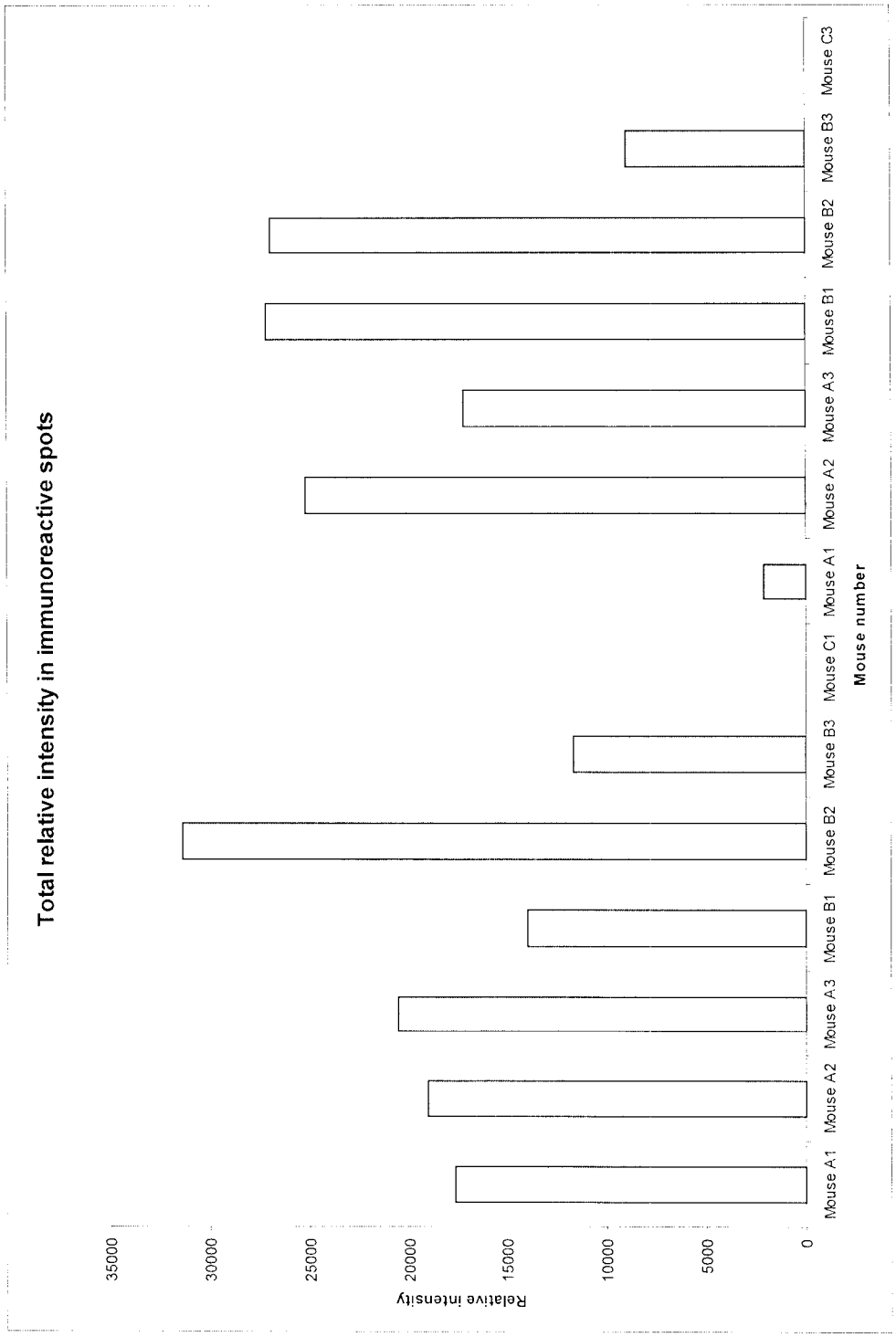

Total relative intensity in immunoreactive spots
Relative intensity
35000
30000
25000
20000
15000
10000
5000
0
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C1
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C3
Mouse number

FIG. 19B

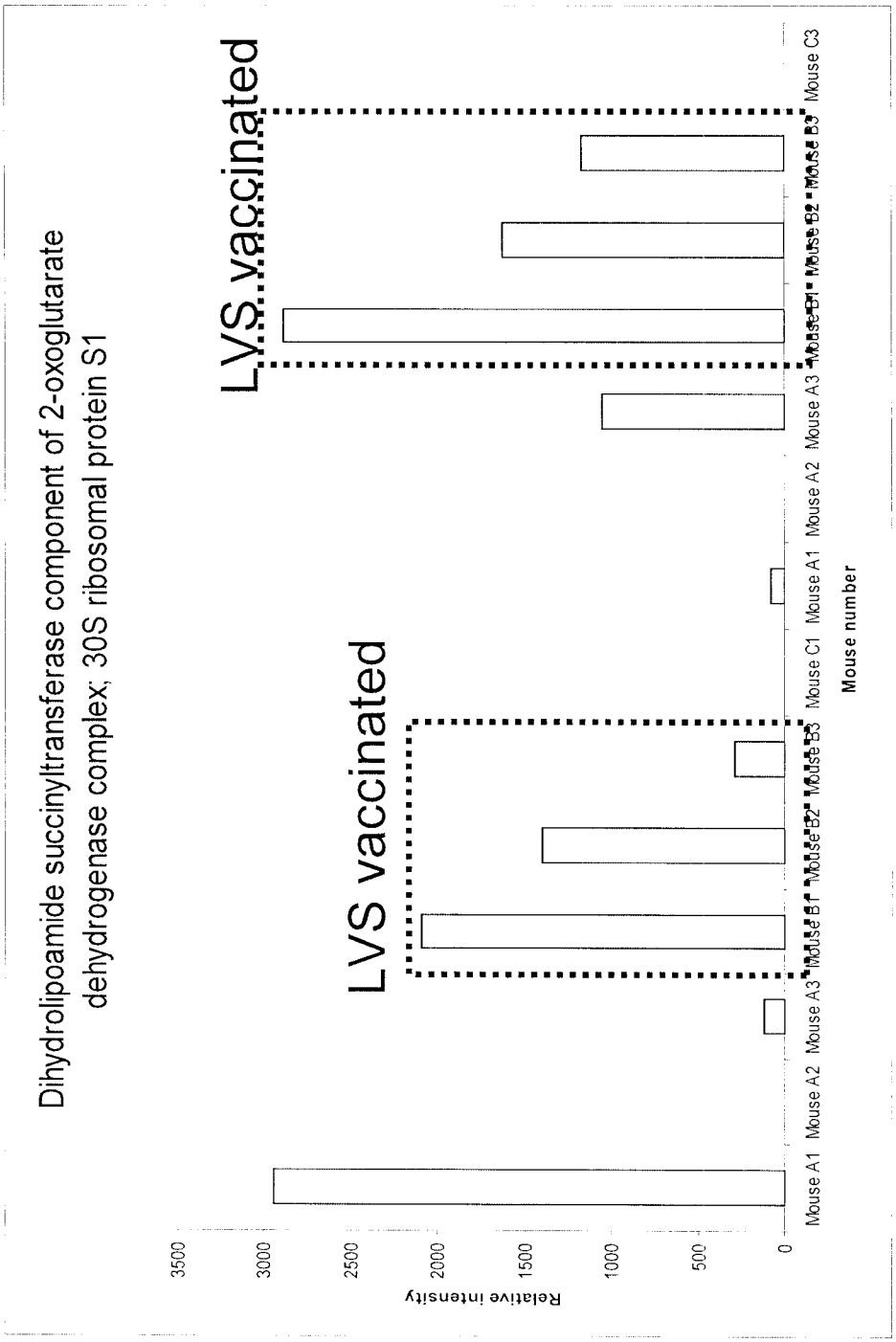

Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex; 30S ribosomal protein S1
LVS vaccinated
LVS vaccinated
Relative intensity
3500
3000
2500
2000
1500
1000
500
0
Mouse A1
Mouse A2
Mouse A3
Mouse C1
Mouse A1
Mouse A2
Mouse A3
Mouse C3
Mouse number

FIG. 19C

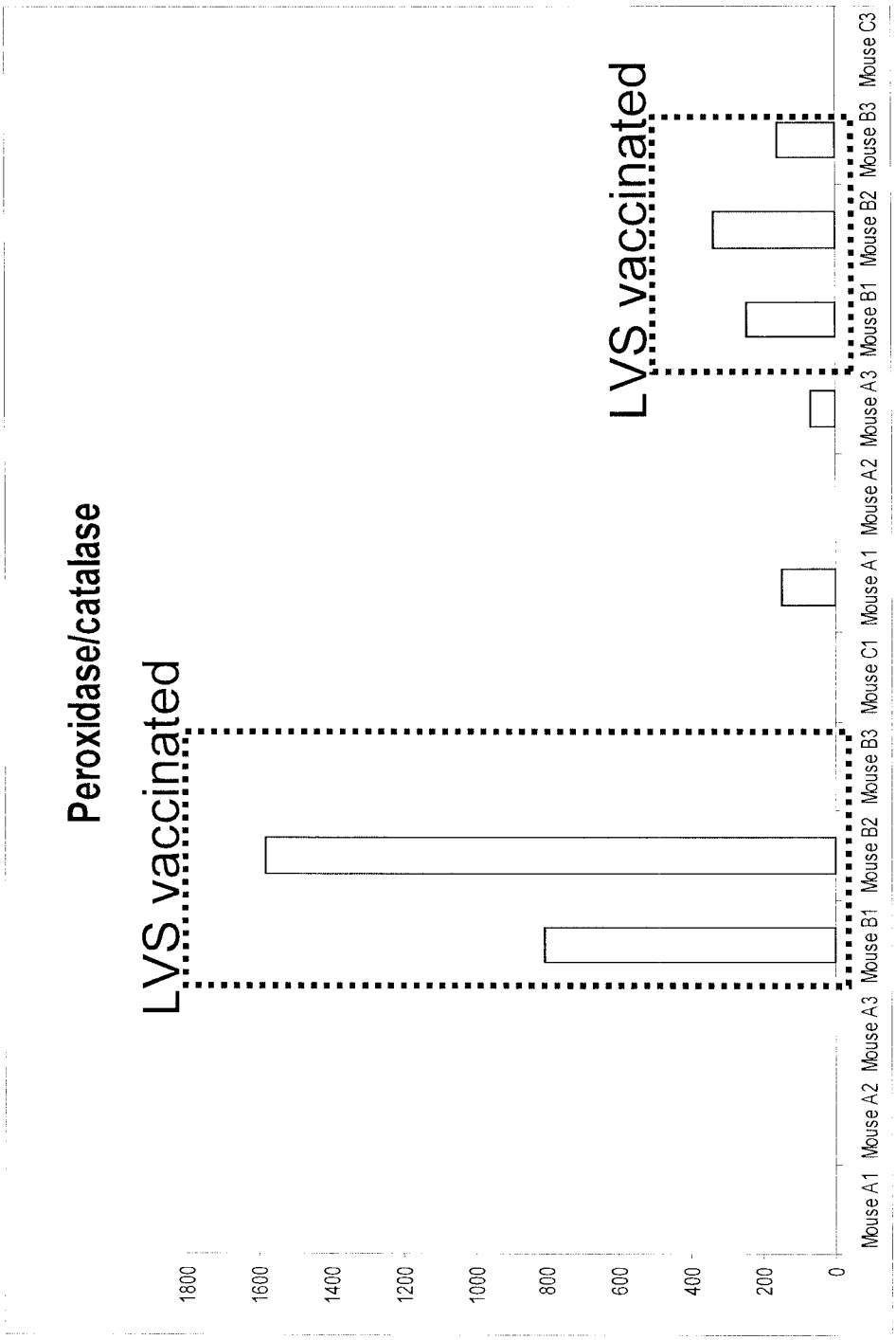

Peroxidase/catalase
LVS vaccinated
LVS vaccinated
1800
1600
1400
1200
1000
800
600
400
200
0
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C1
Mouse A1
Mouse A2
Mouse A3
Mouse B1
Mouse B2
Mouse B3
Mouse C3

FIG. 19D

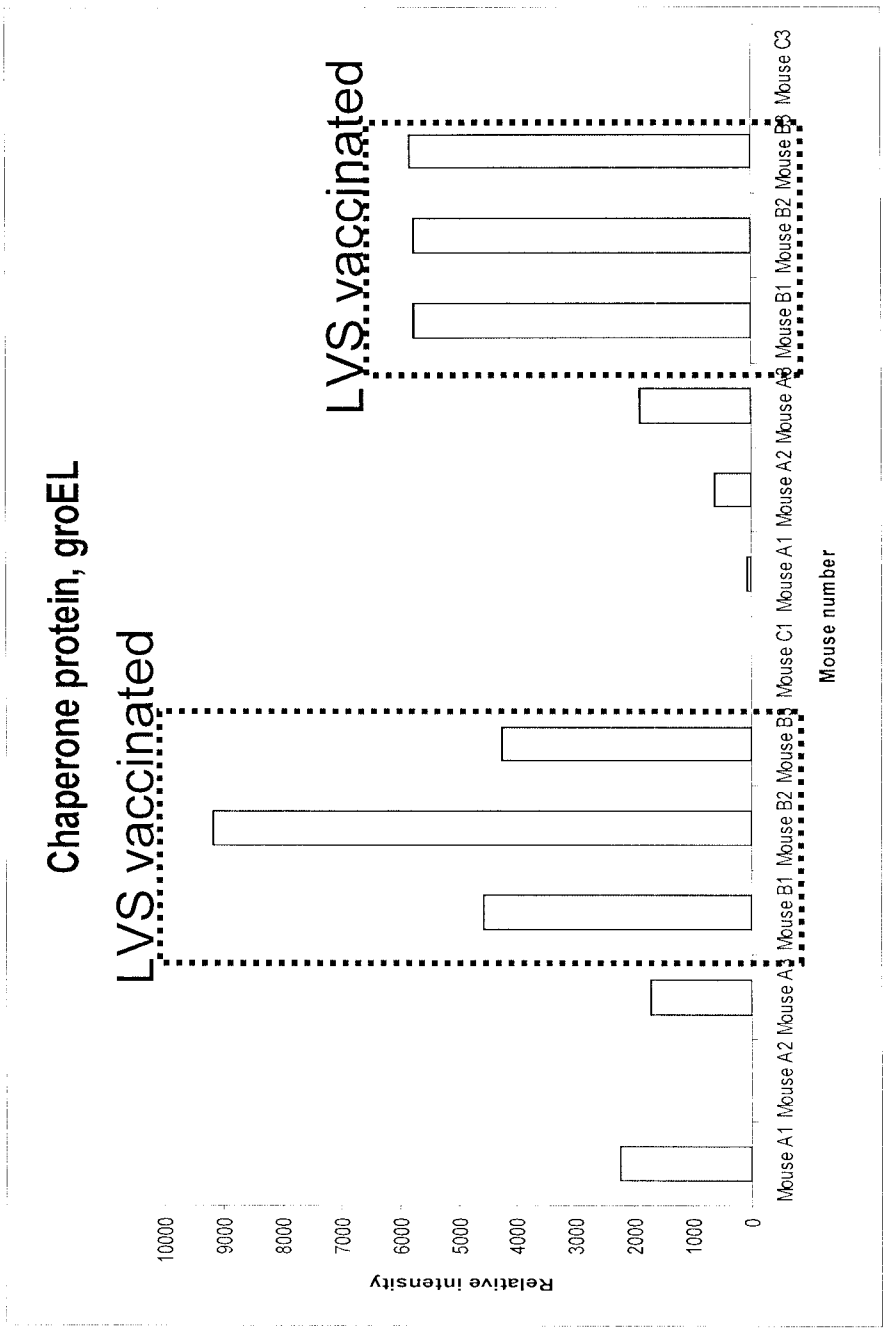

Chaperone protein, groEL
LVS vaccinated
LVS vaccinated
Relative intensity
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Mouse A1 Mouse A2 Mouse A3 Mouse B1 Mouse B2 Mouse B3 Mouse C1 Mouse A1 Mouse A2 Mouse A3 Mouse B1 Mouse B2 Mouse B3 Mouse C3
Mouse number

FIG. 19E

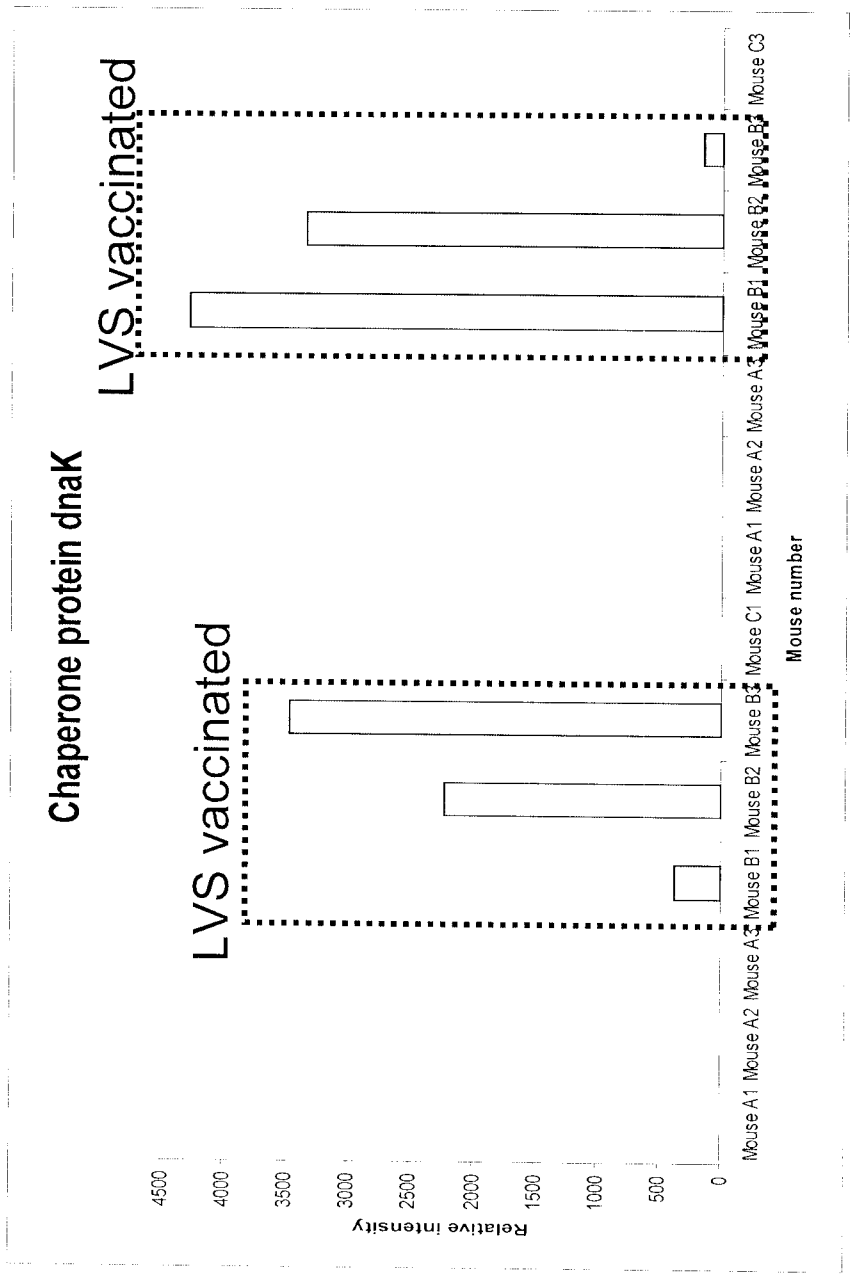

Chaperone protein dnaK
LVS vaccinated
LVS vaccinated
Relative intensity
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mouse A1 Mouse A2 Mouse A3 Mouse B1 Mouse B2 Mouse B3 Mouse C1 Mouse A1 Mouse A2 Mouse A3 Mouse B1 Mouse B2 Mouse B3 Mouse C3
Mouse number

FIG. 19F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000031

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C40B 40/10*** (2006.01) , ***C07K 14/195*** (2006.01) , ***C40B 30/04*** (2006.01) , ***G01N 33/543*** (2006.01) , ***G01N 33/569*** (2006.01)

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 (2006.01): C40B 40/10, C07K 14/195, C40B 30/04, G01N 33/543, G01N 33/569

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: PubMed, SCOPUS, EPODOC, Canadian Patent Database

KW: tularaemia, tularemia, tularensis, biomarker, marker, antigen, FTT0472, FTT0583, FTT1269, FTT1696, FTT1778, FTT0077, FTT1484, FTT0143, FTT0183, FTT0350 and FTT0721

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TITBALL et al. *Francisella tularensis* genomics and proteomics. Annals of the New York Academy of Sciences. June 2007 (06-2007), Vol. 1105, Pages 98-121, ISSN 0077-8923. *Abstract; pages 115-118 "Immunoproteomics" section; and Table 3*	1-12
A	JANOVSKÁ et al. Identification of immunoreactive antigens in membrane proteins enriched fraction from *Francisella tularensis* LVS. Immunology Letters. 15 February 2007 (15-02-2007), Vol. 108, Pages 151-159, ISSN 0165-2478. *Abstract; Materials and Methods; and Tables 1 and 3*	1-12
A	SAVITT et al. *Francisella tularensis* infection-derived monoclonal antibodies provide detection, protection, and therapy. Clinical and Vaccine Immunology. March 2009 (03-2009), Vol. 16, No. 3, Pages 414-422, ISSN 1556-6811. *Abstract; Table 1; and page 418 right column, first paragraph *	1-12
A	TWINE et al. Immunoproteomics analysis of the murine antibody response to vaccination with an improved *Francisella tularensis* live vaccine strain (LVS). PLoS One. 2 April 2010 (02-04-2010), Vol. 5, No. 4, e10000, ISSN 1932-6203. *Abstract; page 2 right column, third paragraph - page 3 left column, last paragraph; Table 1; and page 6 right column, last paragraph*	1-12

[ ] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

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

"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
13 March 2012 (13-03-2012)	19 April 2012 (19.04.2012)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Pascal Lachance (819) 994-8889

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000031

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

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

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purposes of search

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

3. Additional comments :

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000031

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. [X] Claim Nos. : 1-12 (all partially)
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 :

See extra sheet.

3. [ ] Claim Nos. :
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 :

See extra sheet.

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

2. [X] 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 claim 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 claim 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.

Continuation of Box No. II.2:

Claims 1-12 relate to sets of one or more of the tularaemia biomarkers specified in claim 1 and their use for evaluating immunity against tularaemia and for evaluating vaccine efficiency. The combinatorially large number of possible one or more tularaemia biomarker combinations defined by the claims are not supported by the description and therefore fail to comply with the requirements of **Article 5 of the PCT** and **Article 6 of the PCT** to such an extent that a meaningful search over the entire claimed scope is not possible.

An effort was nevertheless made by the ISA to identify subject matter on which a meaningful search could be performed. In view of the description, the subject matter apparently most important for the applicant is the set comprising all of the 11 biomarkers specified in claim 1. The scope of the search and examination was therefore limited to the subject matter of claims 1-12 (all partially), insofar as they relate to a set of tularaemia biomarkers comprising all of the 11 biomarkers specified in claim 1.

Continuation of Box No. III:

Claims 1-12 are directed to a plurality of inventions, wherein each group of invention features one of the specific combinations of one or more tularaemia biomarkers selected from: dihydrolipoamide succinase component of 2-oxoglutarate dehydrogenase complex (FTT_0077), 50S ribosomal protein L7/L12 (FTT_0143), 30S ribosomal protein S1 (FTT_0183), DNA-directed RNA polymerase alpha subunit (FTT_0350), Acetyl-CoA carboxylase (FTT_0472), Outer membrane associated protein fopA (FTT_0583), Peroxidase/catalase (FTT_0721c), Chaperone protein dnaK (FTT_1269c), Pyruvate dehydrogenase E2 component (FTT_1484c), Chaperone protein groEL (FTT_1696c) and Hypothetical membrane protein (FTT_1778c).

The claims are directed to combinations of 11 tularaemia biomarkers and methods relating thereto for evaluating immunity against tularaemia and for evaluating vaccine efficiency. As each of the biomarkers have distinct structural features, the only feature which might serve to unify the claims is the use of combinations of one or more tularaemia biomarkers for evaluating immunity against tularaemia and for evaluating vaccine efficiency.

However, sets of one or more tularaemia biomarkers, including sets comprising one or more of the biomarkers specified in the instant claims, and their use for evaluating immunity against tularaemia and for evaluating vaccine efficiency are known in the art (e.g. see TITBULL et al, JANOVSKÁ et al, SAVITT et al and TWINE et al) and thus the claims do not relate to a single inventive concept *a posteriori* under **Rule 13.1 of the PCT**. Therefore, the claims are directed to a plurality of alleged inventions wherein each specific combination of one or more of the tularaemia biomarkers featured in claims 1-12 represent a plurality of separate inventions.

The claims must be limited to one inventive concept as set out in **Rule 13 of the PCT**.