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(71) Demandeur/Applicant:
CAMRIS INTERNATIONAL, INC., US

(72) Inventeur/Inventor:
AGUIAR, JOAO CARLOS, US

(74) Agent: MBM INTELLECTUAL PROPERTY LAW LLP

(54) Titre : NOUVEL ANTIGENE DESTINE A ETRE UTILISE DANS UN VACCIN ANTIPALUDIQUE
(54) Title: NOVEL ANTIGEN FOR USE IN MALARIA VACCINE

(57) Abrégé/Abstract:
The present invention provides polypeptides useful as antigens expressed at the pre-erythrocytic stage of the malaria parasite. The antigens can be utilized to induce an immune response and sterile protection against malaria in a mammal by administering the antigens in vaccine formulations or expressing the antigens in DNA or other recombinant protein expression systems delivered as a vaccine formulation.

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(71) Applicant: **CAMRIS INTERNATIONAL, INC.** [US/US]; 3 Bethesda Metro Center, Sutie 1600, Bethesda, MD 20814 (US).

(72) Inventor: **AGUIAR, Joao, Carlos**; 10409 Overgate Place, Potomac, MD 20854 (US).

(74) Agent: **KELLY, Kristina, Caggiano**; Duane Morris LLP, 505 9th Street, Suite 1000, Washington, DC 20004 (US).

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(54) Title: NOVEL ANTIGEN FOR USE IN MALARIA VACCINE

(57) Abstract: The present invention provides polypeptides useful as antigens expressed at the pre-erythrocytic stage of the malaria parasite. The antigens can be utilized to induce an immune response and sterile protection against malaria in a mammal by administering the antigens in vaccine formulations or expressing the antigens in DNA or other recombinant protein expression systems delivered as a vaccine formulation.

NOVEL ANTIGEN FOR USE IN MALARIA VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) from United States Provisional Patent Application Ser. No. 62/296,464 filed February 17, 2016, the entirety of which is hereby incorporated by reference herein.

BACKGROUND

[0002] Despite years of effort, a licensed malaria vaccine is not available. One of the obstacles facing the development of a malaria vaccine is the extensive heterogeneity of many of the malaria vaccine antigens. Potential vaccine antigens that have been evaluated in people thus far have not elicited a protective immune response.

[0003] Malaria kills approximately 863,000 people every year. Although a variety of anti-malarial drugs exist, the cost of these drugs can be prohibitive in the relatively poor areas of the world where malaria is endemic. The widespread use of the most commonly employed drugs has also resulted in the expansion of drug-resistant parasites, rendering many of these drugs ineffective. In the absence of inexpensive, highly potent drugs, vaccination represents the most cost-effective way of supplementing traditional malaria interventions.

[0004] A successful malaria vaccine will need to protect people against a large population of antigenically diverse malaria parasites. A vaccine based on a single isolate of a single antigen may not be able to elicit an immune response that is broad enough to protect individuals against this heterogeneous population. One way to potentially enhance the efficacy of antigen-based vaccines, or any other subunit malaria vaccine, would be to incorporate additional malaria antigens into the vaccine, thereby broadening the immune response elicited by the vaccine.

[0005] Malaria vaccine development efforts have focused almost exclusively on a handful of well-characterized *Plasmodium falciparum* antigens. Despite dedicated work by many researchers on different continents spanning more than half a century, a successful malaria vaccine remains elusive. Sequencing of the *P. falciparum* genome has revealed more than five thousand genes, but has given no indication which of these five thousand genes will be useful, or how to identify potential vaccine targets.

[0006] Malaria is caused by mosquito-borne hematoprotozoan parasites belonging to the genus *Plasmodium*. Four species of *Plasmodium* protozoa (*P. falciparum*, *P. vivax*, *P.* ovale and *P.* malariae) are responsible for the disease in humans. Others cause disease in animals, such as *P.* yoelii and *P.* berghei. *P. falciparum* accounts for the majority of infections and deaths in humans. Malaria parasites have a life cycle consisting of four separate stages. Each one of these stages is able to induce specific immune responses directed against the parasite and the correspondingly occurring stage-specific antigens, yet naturally induced malaria does not protect against reinfection.

[0007] Malaria parasites are transmitted to mammals by several species of female Anopheles mosquitoes. Infected mosquitoes deposit the sporozoite form of the malaria parasite into the mammalian skin during a blood meal, which subsequently invades the bloodstream. Sporozoites remain for a few minutes in the circulation before invading hepatocytes. At this stage, the parasite is located in the extra-cellular environment and is exposed to antibody attack, mainly directed to the circumsporozoite (CS) protein, a major component of the sporozoite surface. Once sporozoites invade hepatocytes, the parasite differentiates, replicates and develops into a schizont. During this stage, the invading parasite will undergo asexual multiplication, producing up to 20,000 daughter merozoites per infected hepatocyte cell. During this intra-

cellular stage of the parasite, the host immune response includes T lymphocytes, especially $CD8^+$ T lymphocytes. After 10-14 days of liver infection, thousands of newly formed merozoites are released into the bloodstream and invade red blood cells (RBCs), becoming targets of antibody-mediated immune response and T-cell secreted cytokines. After invading the erythrocytes, the merozoites undergo several stages of replication, transforming into trophozoites, and schizonts, which rupture to produce a new generation of merozoites that subsequently infect new RBCs. This phase (erythrocytic) of the parasite stimulates a strong humoral response that can block merozoite invasion of RBCs and usually confers protection against pathology associated with this phase. The erythrocytic stage is associated with overt clinical disease. A smaller number of trophozoites may develop into male or female gametocytes, which are the parasite's sexual stage. When susceptible mosquitoes ingest gametocytes, the fertilization of these gametes leads to zygote formation and subsequent transformation into ookinetes, then into oocysts, and finally into sporozoites, which migrate to the salivary gland to complete the cycle.

[0008] The two major arms of the pathogen-specific immune response that occur upon entry of the parasite into the body are cellular and humoral. The one arm, the cellular response, relates to $CD8^+$ and $CD4^+$ T cells that participate in the immune response. Cytotoxic T lymphocytes (CTLs) are able to specifically kill infected cells that express pathogenic antigens on their surface. CD4+ T cells or T helper cells support the development of CTLs, produce various cytokines, and also help induce B cells to divide and produce antibodies specific for the antigens. During the humoral response, B cells specific for a particular antigen become activated, replicate, differentiate and produce antigen-specific antibodies.

[0009] Both arms of the immune response are relevant for protection against a malarial infection. When infectious sporozoites travel to the liver and enter the hepatocytes, the

sporozoites become intracellular pathogens, spending little time outside the infected cells. At this stage, $CD8^+$ T cells and $CD4^+$ T cells are especially important because these T cells and their cytokine products, such as interferon-γ (IFN-γ), contribute to the killing of infected host hepatocytes. Elimination of the intracellular liver parasites in the murine malaria model is found to be dependent upon $CD8^+$ T cell responses directed against peptides expressed by liver stage parasites. Depletion of $CD8^+$ T cells abrogates protection against sporozoite challenge, and adoptive transfer of $CD8^+$ T cells to naïve animals confers protection.

[0010] When a malarial infection reaches the erythrocytic stage in which merozoites replicate in RBCs, the merozoites are also found circulating freely in the bloodstream for a brief period until they invade new erythrocytes. Because the erythrocyte does not express either Class I or II MHC molecules required for cognate interaction with T cells, it is thought that antibody responses against the parasite are most relevant at the blood stage of the parasite lifecycle. In conclusion, a possible malaria vaccine approach would be most beneficial if it would induce a strong cellular immune response as well as a strong humoral immune response to tackle the different stages in which the parasite occurs in the human body.

[0011] Current approaches to malaria vaccine development can be classified according to the different developmental stages of the parasite, as described above. Three types of possible vaccines can be distinguished. The first is pre-erythrocytic vaccines, which are directed against sporozoites and/or schizont-infected hepatocytes. Historically, this approach has been dominated by (CSP)-based strategies. Since the pre-erythrocytic phase of infection is asymptomatic, the goal of a pre-erythrocytic vaccine would be to confer sterile immunity, mediated by humoral and cellular immune response, and thereby prevent latent malaria infection. This goal has not been met by any known treatment.

[0012] The second type of vaccine approach is asexual blood stage vaccines, which are directed against either the infected RBC or the merozoite itself, are designed to minimize clinical severity or prevent infection if antibodies prevent merozoites invading erythroctyes. Attempts to create such vaccines so far have failed to sufficiently reduce morbidity and mortality or prevent the parasite from entering and/or developing in the erythrocytes. Transmission-blocking vaccines are designed to hamper the parasite development in the mosquito host. Attempts to create this type of vaccine so far have failed to reduce population-wide malaria infection rates.

[0013] The final type of vaccine approach is combination malaria vaccines that target multiple stages of the parasite life cycle. This approach attempts to develop multi-component and/or multi-stage vaccines. Attempts to create such vaccines so far have failed to effect sufficient protection. As a result of these failures, there is currently no commercially available vaccine against malaria.

[0014] Immunization of rodents, non-human primates, and humans with radiation-attenuated sporozoites (RAS) has been found to confer protection against a subsequent challenge with viable sporozoites. However, the expense and the lack of a feasible large-scale culture system for the production of irradiated sporozoites, the relative short-term efficacy, lack of cross-strain protection, and the need to be delivered intravenously have been obstacles to the development of such vaccines.

[0015] The CS protein is the only *P. falciparum* antigen demonstrated to prevent malaria infection when used as the basis of active immunization in humans against mosquito-borne infection. The protection levels for this antigen, however, are not high enough to support a viable therapy. In theory, vaccine protection levels should be above 85% in order to be a viable therapy. With protection lower than that, mutants that are more virulent may escape in endemic

areas. CS antigen-based vaccines have demonstrated an efficiency of only about 50% and that protection does not last more than a year. Nevertheless, this is still the best known antigen response prior to the present disclosure.

[0016] The entire genomic sequence of *P. falciparum* has been sequenced. *See* Bowman et al., Nature, 400: 532-538 (1999); Gardner, et al., Nature, 419: 498-511 (2002). Another human malaria parasite, *P. vivax*, has also been sequenced. *See* Carlton et al., Nature, 455: 757-763 (2008). The rodent malaria parasite, *P. yoelii* has also been sequenced. *See* Carlton et al., Nature, 419: 512-519 (2002). Despite this, however, the development of efficacious anti-malaria vaccines has been severely hampered by the inability to identify promising antigens. Sequencing of the *P. falciparum*, *P. vivax*, and *P. yoelii* genomes has resulted in the identification of 5,369, 5,433, and 5,675 genes, respectively. Knowledge of these sequences alone, however, will not likely result in new vaccine constructs. Consequently, only 0.2% of the *P. falciparum* proteome is undergoing clinical testing, and these tests have failed to induce high grade protection in volunteers.

SUMMARY

[0017] The present invention provides polypeptides useful as antigens that are expressed at both the pre- and erythrocytic stage of the malaria parasite. The antigens can be utilized to induce both cellular and humoral immune responses against malaria in a mammal by administering the antigens in vaccine formulations or expressing the antigens in DNA or other nucleic acid expression systems delivered as a vaccine formulation. In preferred embodiments, the mammal is a human.

[0018] In one preferred embodiment, the invention provides an immunogenic composition for protecting a mammal against malaria infection, the immunogenic composition

comprising one or more recombinant polypeptides of SEQ ID NO. 3 or SEQ ID NO. 6, or derivatives thereof in a pharmaceutically acceptable carrier. In general, derivatives have at least 10 contiguous amino acids of and/or 85% identity with the reference sequence. The immunogenic composition can be formed from an isolated or recombinant polypeptide or a carrier virus expressing the recombinant antigen and may be paired with an acceptable adjuvant.

[0019] The antigens that are the subject of the present disclosure are identified by different nomenclatures in different contexts, as is standard in this art. For convenience, the table below identifies each antigen by its sequence, as well as the various names and shorthands used in the prior art and in the disclosure herein:

Shorthand	**PlasmoDB Identification**	**SEQ ID NO.**
Py E140	PY06306, PY17X_0210400, PYYM_0211900	1 (amino acid) 2 (nucleotide)
Pf E140	PFA0205w, MAL1P1.31, PF3D7_0104100, XP_001350973	3 (amino acid) 4 (nucleotide)
Pv E140	PVX_081555, PV081555, PVP01_0210600	6 (amino acid) 5 (nucleotide)
Py falstatin	PY17X_0816300, PY03424, PYYM_0816000	
PyCSP	PY03168, PYYM_0405600	
Py E057	PY03396, PY17X_1006600, PYYM_1006600	
Py E137	PY05693, PY17X_1006100, PYYM_1006100	
Py UIS3	PY03011, PY17X_1402400	
Pf falstatin, ICP	PFI0580C or PF3D7_0911900	7 (amino acid)

Pf CSP	PFC0210C, MAL3P2.11, PF3D7_0304600	8 (amino acid)
Pf UIS3, ETRAMP13	PF13_0012, PF3D7_1302200	9 (amino acid)

[0020] The invention may comprise a combination of two or more recombinant polypeptides in a pharmaceutically acceptable carrier, wherein one polypeptide is SEQ ID NO. 3, SEQ ID NO. 6, or derivatives thereof, and the other polypeptide is any of the *falciparum* or *vivax* orthologs of PyCSP, Py falstatin, Py UIS3, PY03396, PY05693, PY03424, and PY03011.

[0021] The present invention also includes a method of inducing an immune response against malaria in a mammal by administering an immunologically effective amount of a composition comprising one or more polypeptides encoded by SEQ ID NO. 3 or 6, or derivatives thereof. Alternatively, the method may include administering one or more priming or boosting immunizations against malaria, wherein said priming and boosting immunizations comprise an immunologically effective amount of an recombinant polypeptide as described. The method of administering the polypeptide can include use of a suitable expression vector, such as a plasmid, replicating viral vector, or nonreplicating viral vector. A suitable expression vector can be a DNA plasmid, baculovirus, rVSV, SpyVLPs, alphavirus replicon, adenovirus, poxvirus, adenoassociated virus, cytomegalovirus, canine distemper virus, yellow fever virus, retrovirus, RNA replicon, DNA replicon, alphavirus replicon particle, Venezuelan Equine Encephalitis virus, Semliki Forest Virus, or Sindbis Virus.

[0022] The polypeptides useful as antigens disclosed herein are the first *Plasmodium* pre-erythrocytic antigens that can sterilely protect 100% of subjects against an infectious *P. yoelii* sporozoite challenge. These responses are conveniently measured in mice as a proxy for their human orthologs. Malaria infection, treatment, and immunity has been studied extensively in

both mice and humans, and mouse models are considered a standard indicator of malaria vaccine efficacy in human and other mammalian subjects. The PY06306 antigen disclosed herein alone protects 71% to 100% of CD1 mice against malaria and in addition induces an immune response capable of delaying the parasite onset in the blood of remaining non-protected mice. Overall, 83% (384/461) of PY06306-immunized mice were protected from malaria infection. This protection is reported for both outbred (CD1) and inbred (BABB/c) strains of mice, using a rigorous 300- and 100-sporozoite challenge, respectively, and efficacy assessment as sterile protection. The efficacy of the antigen disclosed herein, in light of the relationships among murine, primate, and human malaria immune responses disclosed herein, and standard indicators of vaccine efficacy, presents a polypeptide for inducing an immune response against malaria in a mammal.

BRIEF DESCRIPTION OF DRAWINGS

[0023] Figure 1 shows the protection results for a matrix experiment in which fourteen CD1 outbred mice per group were immunized in a prime-boost regimen with a combination of DNA and Human Adenovirus type 5 (Ad5) vectors that express PY03396, PY05693, PY06306, PY00232 and PyCelTOS. Positive control mice were immunized with DNA and Ad5 vectors that express PyCS*P*. Negative control mice were immunized with 4X relative amount of DNA and Ad5 vector that do not express *P. yoelii* antigen and naïve mice. Gray and black bars indicate antigen combination groups with and without PyCSP, respectively. Hatched and checkered bars represent PyCSP and naïve groups, respectively. The mice were challenged with 300 *P. yoelii* sporozoites and evaluated for parasitaemia by examining Giemsa-stained blood smears up to 14 days post challenge. Numbers at bottom denote number of sterile protected mice per total challenged mice in each group.

[0024] Figure 2 shows a matrix deconvolution of the experiment evaluating PY06306 and other antigens shown illustrated in Figure 1. Fourteen CD1 outbred mice per group were immunized in a prime-boost regimen comprising of DNA and Adenovirus type 5 (Ad5) vectors that express PY03396, PY05693, PY06306, PY03424 and PY03011. Positive control mice were immunized with DNA and Ad5 vectors that express PyCSP. Negative control mice were immunized with 4X relative amount of DNA and Ad5 vectors that do not express *P. yoelii* antigen. Gray and black bars indicate antigen combination groups with and without PyCSP, respectively. Hatched and checkered bars represent PyCSP and null-immunized mice, respectively. The mice were challenged with 300 *P. yoelii* sporozoites and evaluated for parasitaemia by examining Giemsa-stained blood smears up to 17 days post challenge. Numbers at bottom denote number of sterile protected mice per total challenged mice in each group.

[0025] Figure 3 shows a Kaplan-Meier curve depicting the percentage of protected mice for the time to parasitemia after challenge. Data extracted and analyzed from matrix deconvolution experiment 2. Closed circles indicate CD1 mice immunized with PY06306 antigen alone, Symbols Xs, squares and triangles indicate PyCSP, 4X Null and Naïve mice, respectively. The mice were challenged with 300 *P. yoelii* sporozoites and evaluated for parasitaemia by examining Giemsa-stained blood smears up to 14 (PyCSP, 4X Null and Naïve) or 17 (PY06306) days post challenge.

[0026] Figure 4 shows antibody responses for the matrix deconvolution experiment. Endpoint immunofluorescence assay (IFA) titers were measured on *P. yoelii* sporozoite and blood stage parasites. Sera collected one week after Adeno 5 boost was pooled per group of antigen combination and assayed for reactivity on air-dried parasites. Black and gray bars indicate sporozoite and blood stages reactivity, respectively. Positive control antibodies were

NYS1 and NYLS3 monoclonal antibodies, respectively. Sera from 4X null and naïve animals were negative.

[0027] Figure 5 shows antibody titers of protected and non-protected mice for the matrix deconvolution Experiment shown in Figure 4. Endpoint Immunofluorescence (IFA) titers were measured against *P. yoelii* sporozoite for individual mice for six PY06306 (E140)-containing groups of mice. One group from matrix experiment 2 (Mx2); E140, E137, E057 combination (closed circles) and five groups from matrix deconvolution experiment 2 (MDx2); E140, E137, E057 combination (closed squares), E140 alone (closed diamonds), E140, E137 combination (closed stars), E140, E057 combination (closed triangles), and E140, E137, E057, PY3424 combination (closed asterisks). All protected mice are displayed by closed symbols and all non-protected by the X symbol. Mann-Whitney non-parametric test indicates statistical significance;**, $p<0.005$ and***, $p=0.001$.

[0028] Figure 6 shows continued protection at 11 weeks for the deconvolution study shown in Figure 2. Sterilely protected mice were rested for 11 weeks and then challenged with 200 P. *yoelii* sporozoites. Protection was measured by examining Giemsa-stained blood smears up to 17 days post challenge.

[0029] Figure 7 shows the PY06306 (Py E140) antigen homology among *Plasmodium spp*, including Pf (human *P. falciparum*), Pv (human *P. vivax*), Pc (rodent *P. chabaudi*), Py (rodent *P. yoelii*), Pb (rodent *P. berghei*), Pk (primate *P. knowlesi*), Pr (primate *P. rhodiani*), and Pg (primate *P. gaboni*).

[0030] Figure 8 shows the PY06306 (Pf E140) (PFA0205w or MAL1P1.31 or PF3D7_0104100) amino acid conservation among various Pf parasite strains. These parasites

were collected from a variety of countries in different continents. The highest (99%) and the lowest (92%) homology are highlighted.

[0031] Figure 9 shows the results of an in vivo T cell depletion experiment in mice. CD1 outbred mice were immunized with PY06306 DNA and boosted with Adeno 5 vaccines, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ T cells depleted (black bars) before and after challenge with 300 *P. yoelii* sporozoites. Rat Ig and no depletion groups were used as positive controls. Groups of null-immunized mice (gray bars) were also depleted the same way and used as negative controls. PyCSP (diagonal bar) and Naïve (stripe bar) were experimental positive and negative controls. Arrows indicate the type of depletion and the number of mice sterile protected out of the number immunized. Challenged mice were evaluated for parasitaemia by examining Giemsa-stained blood smears up to 19 days post challenge.

[0032] Figures 10A and 10B shows sera transfer studies in CD1 and BALB/c mice. In Figure 10A, groups of 14 BALB/c mice were either immunized with DNA/Adeno virus 5 encoding PY06306 (solid black line) and PyCSP (solid gray line). Sera from immunized and non-challenged mice were collected and transferred 24 and 6 hours before challenge to naïve recipient mice; PY06306 (dotted black line) and PyCSP (dotted gray line). After challenge with 300 *P. yoelii* sporozoites, mice were monitored for parasitaemia for 17 days. In Figure 10B, groups of 14 CD1 mice were either immunized with DNA/Adeno virus 5 encoding PY06306 (solid black line) and PyCSP (solid gray line). Sera from immunized and non-challenged mice were collected and transferred 24 and 6 hours before challenge to naïve recipient mice; PY06306 (dotted black line) and PyCSP (dotted gray line). After challenge with 100 *P. yoelii* sporozoites, mice were monitored for parasitaemia for 17 days. Percentage of sterilely protected mice for each group is shown in the legend box.

[0033] Figure 11 shows PY06306 protection against a blood stage challenge. Fourteen CD1 mice per group were immunized with a dose of DNA and boosted with Adenovirus 5 expressing PY06306 (black bar), PY06306 + PyFalstatin (gray bar), and PyFalstatin alone). Null-immunized and naïve were used as negative control groups of mice. PyFalstating is also known as PY03424. All mice were challenged with 10,000 infected *P. yoelii*-infected erythrocytes and parasitaemia monitored for 17 days after challenge by Giemsa-stained thin smears.

[0034] Figure 12 shows protection with mammalian codon-optimized Adenovirus 5 in a chart comparing native (na) and codon-optimized (co) PY06306 and route of immunizations. CD1 mice (14 per group) were primed with a co E140 DNA and boosted with either native PY06306 Adeno 5 (black bars) or mammalian co PY06306 Adeno 5 (gray bars). Both Adeno 5 constructs were administered intramuscular (IM) in decreasing doses from 10^10, 10^9, 10^8, and 10^7 PU. Two additional groups of mice were boosted with Ad5 administered either subcutaneously (SC) or intravenously (IV). Two additional mice groups were not primed with DNA vaccine and instead immunized with a single IM dose of either na or co PY06306 Ad5 two weeks before challenge. Null-immunized (stripe bar) and Naïve (checkered bar) mice are negative controls. All mice were challenge with 300 *P. yoelii* sporozoites, parasitaemia were monitored over 18 days by thin blood smears stained with Giemsa.

[0035] Figure 13 shows that Pf E140 (PFA0205w or MAL1P1.31 or PF3D7_0104100) is immunogenic in mice. IFA titers induced by PFA0205w vaccines. Both CD1 and BALB/c mice were immunized with PFA0205w (PfE140) vaccines reagents: DNA vaccine in VR1020-DV plasmid, Adenovirus 5, and full length recombinant protein expressed by the wheat germ system as GST and 6xHis fusions. Recombinant proteins were emulsified in Montanide ISA 720

adjuvant and immunized SC as 5 μg/dose. Immunofluorescence (IFA) titers were measured against both *P. falciparum* sporozoites and a mixture of several of blood stages.

[0036] Figure 14 shows that the *P. falciparum* E140 (PFA0205w) is naturally immunogenic in humans. T cell responses to PFA0205w (PfE140 or PF3D7_0104100) by *P. falciparum* radiation attenuated sporozoites (RAS)-immunized human subjects. PBMCs were stimulated with overlapping 15mer peptide PFA0205w pools A for 21h with brefeldin A and stained for viability, phenotypic (CD14, CD19, CD3, CD4, and CD8), and intracellular functional markers (including IFN-γ and CD154). The background subtracted frequencies of $CD4^+$ T cells producing IFN-γ and intracellular CD154 (A) and $CD8^+$ T cells producing IFN-γ(B) are shown. Positive responses for PFA0205w pool A (filled symbols) in both experiments were identified as those exceeding two standard deviations from the average of the negative control (DMSO stimulated) samples.

[0037] Figure 15 shows that PVX_081555 (PvE140) is relatively abundant in *P. vivax* sporozoites. 256 *P. vivax* sporozoite proteins sequenced using multi-dimensional-protein-identification-technology (MudPIT) were graphed based on their relative abundance defined by their quantitative value. The positions of *P. vivax* circumsporozoite protein and *P. vivax* E140 (PVX_081555) are indicated in the graph with a black arrow.

DETAILED DESCRIPTION

[0038] The inventor has determined that pre-erythrocytic proteins are critical in conferring protective immunity against malaria. Despite the relatively large number of malaria genes that have been identified, following sequencing of the malaria parasite genome, identification of vaccine candidates has been hampered, to a great extent, by the relatively

complex life-cycle of malaria parasite. Furthermore, many genes of the malaria parasite are poorly defined, antigenically, as well as functionally.

[0039] Against this backdrop, the inventor decided to undertake high-throughput screening of antigens encoded by numerous genes in order to ascertain potential protective responses. The inventor developed a novel strategy for identifying and testing potential malaria antigens that overcame the difficulties experienced in the prior art. This novel approach included identifying certain traits that the inventor determined would be indicative of potential human vaccine candidates. The inventor then compiled a list of 146 *P. yoelii* orthologs of *P. falciparum* genes that were believed to possess these traits. The inventor then designed cloning primers, and conceived of a strategy for cloning the genes and screening by transfection ELISpot. The transfection ELISpot involved transfecting an A20 cell line with the VR1020 vaccine constructs, expressing the antigen, and using these transfected cells to present antigens in the ELISpot assay. This use of ELISpot was a novel strategy for screening antigens. Priority antigens were identified from a large panel of *P. falciparum* proteins. The priority antigens were evaluated based on a number of criteria judged by the inventor to be relevant to protection against malaria. One such criterion was selecting antigens that are expressed in the sporozoite and liver stages of the malaria parasite; i.e. pre-erythrocytic antigens. Certain antigens among those selected based on this criterion showed protective responses in mice that indicated that orthologs of those genes in humans would encode human antigens useful as potential vaccine formulations. One gene in particular, PY06306, later curated as PY17X_0210400, which is the subject of this disclosure, surprisingly showed dramatic and consistent protection responses indicating that gene as encoding an antigen for which orthologs would be useful as a leading vaccine formulation.

[0040] The sequence documented for the PY06306 gene, however, was only partial (479 aa) and originated from the early genome annotation. In order to perform the protection experiments disclosed herein with the full-length antigen (816 aa), the inventor needed to re-clone the gene. A similar situation occurred with the *P.falciparum* (human homolog), which also needed to be re-cloned from what was known in the art. The sequences disclosed in the listing provided herein, used in all of the examples, and reflected in all of the data examples conform to the inventor's corrected version of the gene, rather than what was previously believed in the art to be the relevant sequence.

[0041] The invention relates to DNA and amino acid sequences encoding recombinant *Plasmodium falciparum* and *Plasmodium vivax* proteins. Specifically, the invention relates to a highly protective pre-erythtrocytic *Plasmodium yoelii* and its *P. falciparum* and *P. vivax* ortholog antigens for use in a malaria vaccine. The relevant sequences can be utilized to express the encoded proteins for use as subunit immunogenic antigens or can be incorporated into vectors suitable for in vivo expression in a host in order to induce an immunogenic response. The antigens can be utilized in combination or singly in immunogenic formulations.

[0042] In one embodiment, the immunogenic composition is a DNA-based vaccine. DNA was found to be a viable platform for delivering the immunogenic compositions of the present disclosure. A DNA-based vaccine can be delivered by recombinant viruses, such as Modified Vaccinia Ankara (MVA) attenuated poxvirus, Vesicular Stomatitis Virus (VSV), or GC46 (gorilla adenovirus) viruses. Other human Adenovirus alternatives like these can also be used, such as baculovirus.

[0043] In another embodiment, the composition comprises immunogenic proteins. In this embodiment, the proteins can be produced by first inserting the DNA encoding the proteins

in suitable expression systems. These include, for example, Adenoviral based systems, a poxvirus based system, or a DNA plasmid system. The expressed and purified proteins can then be administered in one or multiple doses to a mammal, such as humans. In this embodiment, the purified proteins can be expressed individually or DNA encoding specific proteins can be recombinantly associated to form a single immunogenic composition. These immunogenic compositions can then be administered in one or multiple doses to induce an immunogenic response.

[0044] One embodiment of the invention relates to recombinant polypeptides expressed as full-length or fragments by heterologous expression systems. Examples of such systems are: *Escherichia coli*, yeast (*Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (HEK293 or CHO cells), baculovirus-infected insect cells, and Drosophila S2 stable cells.. The recombinant proteins can be incorporated in immunogenic formulations in order to induce an immune response. In this embodiment, the polypeptides can be incorporated singly or in combination. The immunogenic compositions of the invention can also include adjuvants to improve or enhance the immune response elicited by the polypeptides. Suitable adjuvants include ALFQ, a non-toxic formulation comprising a monophosphoryl lipid A-containing liposome composition with saponin.

[0045] Adjuvants have traditionally been broadly classified into two major classes according to their component sources, physiochemical properties or mechanisms of action, namely: (i) immunostimulants such as TLR ligands, cytokines, saponins and bacterial exotoxins that directly act on the immune system to increase responses to antigens and (ii) vehicles such as mineral salts, emulsions, liposomes, virosomes and biodegradable polymer microspheres that present vaccine antigens and co-administered immununostimulants to the immune system in an

optimal manner. In recent years it has become apparent that many of these vehicles also have a direct effect on the immune system and can be considered immunostimulants.

[0046] Examples of acceptable adjuvants for inclusion with a malaria vaccine include Army Liposome Formulation (ALF) derivatives such as ALF, ALFA (plus aluminum), and ALFQ (plus QS21). Other options include a lipid A derivative and a saponin in a liposome formulation, such as QS21 and 3D- monophosphoryl lipid A (a non-toxic derivative of lipopolysaccharide), other immunostimulants that are similar in structure to LPS, MPL, or 3D-MPL, acylated monosaccharides, saponin derivatives (Quil-A, ISCOM, QS-21, AS02 and AS01), soluble triterpene glycosides, Toll-like receptor 4 (TLR4) agonists, montanides (ISA51, ISA720), immunostimulatory oligonucleotides, and imidazoquinolines. Adjuvants may be prepared in cholesterol-containing liposome carriers.

[0047] As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product. Proteins are included within the definition of polypeptides. The term "mer," in conjunction with a number, such as 15-mer, refers to the length of a polypeptide in numbers of amino acids.

[0048] As used herein, the proteins may be prepared for inclusion of an effective amount of one or more polypeptides described herein into an immunogenic composition by first expressing the appropriate gene fragments by molecular methods, expression from plasmids or other expression systems such as viral systems and then isolated. A further aspect of the invention is the ability of the proteins to induce an humoral and/or T-cell immune response.

[0049] An embodiment of the invention is the incorporation of DNA encoding the polypeptides in vector expression systems, wherein the system permits expression of one or more polypeptides in mammalian host cells, such as in humans to induce an immune response. The

expression systems can be DNA plasmids or viral systems. Methods for preparing and administering a DNA vaccine expressing *Plasmodium* proteins are well known in the art.

[0050] In another embodiment, derivatives of the proteins can be used in immunogenic compositions. In a variant of this embodiment, the immunogenic derivatives of the *P. falciparum* and *P. vivax* proteins include at least 10 contiguous amino acids of an amino acid sequence of a full length polypeptide comprising an amino acid sequence disclosed herein. Immunogenic derivatives of the polypeptides may be prepared by expression of the appropriate gene fragments or by other methods such as by peptide synthesis. Additionally, derivatives may be a fusion polypeptide containing additional sequence encoding one or more epitopes of the *P. falciparum* polypeptides disclosed herein. In these embodiments, the proteins can be directly incorporated in immunogenic formulations or expressed from DNA plasmids or viral expression systems.

[0051] In some embodiments, the *P. falciparum* and *P. vivax* polypeptides include immunogenic derivatives with more than 80% amino acid sequence identity to the sequences disclosed herein. In this context, the term "identity" refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same, when aligned for maximum correspondence. Where sequences differ in conservative substitutions, i.e., substitution of residues with identical properties, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution.

[0052] When the compositions are prepared for administration, they are preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. A "pharmaceutically acceptable carrier" is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the

formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a dry powder or as granules; as a solution, a suspension or an emulsion. The composition exists as dry powder prior to reconstitution in a liquid carrier.

[0053] Pharmaceutical formulations containing the immunogenic compositions of the invention can be prepared by procedures known in the art using well known and readily available ingredients. The therapeutic agents of the invention can also be formulated as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes. The pharmaceutical formulations of the therapeutic agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

[0054] Thus, the immunogenic composition may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The composition is suitable for injection intravenously, subcutaneously, or intramuscularly. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

[0055] Additionally, the immunogenic composition may contain formulatory agents that do not occur naturally in the cellular environment in which the peptide is expressed. Such

formulatory agents include any surfactants, diluents, solubilizers, emulsifiers, buffers, thickeners, preservatives, detergents, adjuvants, excipients, and antimicrobials that do not naturally occur in the cellular environment in which the peptide is expressed, but nonetheless serve to artificially enhance the bioavailability, effectiveness, delivery, storage, administration, absorption, stability, safety, or function of the peptide in the immunogenic composition before, after, or during administration to a mammal.

[0056] Alternately, the immunogenic composition may be provided as a dry powder. A dry powder composition may be prepared by freeze drying, spray drying, and freeze spray drying a solution or suspension containing the polypeptides described herein, and may further optionally include milling or lyophilization with milling. The dry powder may be suitable for direct administration to a patient, such as through inhalation or capsule ingestion, or may be suitable for suspension or reconstitution in a fluid carrier. Dry powder formulations may include physiologically acceptable carrier powders, such as excipients, dispersants, stabilizers, humectants, anti-caking agents, or other additives.

[0057] The immunogenic compositions of the present invention, both dry powder and fluid embodiments, may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing, or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in formulations of the present composition include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions pH 7.0-8.0. The composition of the present disclosure may also comprise combinations of other agents such as diluents, which may include water, saline, glycerol or other suitable alcohols, wetting or emulsifying agents; buffering agents;

thickening agents for example cellulose or cellulose derivatives; preservatives; detergents; antimicrobial agents; and the like.

[0058] Where the immunogenic composition is used as a vaccine, the composition comprises an immunologically effective amount of the peptides described herein. An "immunologically effective amount" of an antigen is an amount that when administered to an individual, either in a single dose or in a series of doses, is effective for treatment or prevention of malaria infection. This amount will vary depending upon the health and physical condition of the individual to be treated and on the antigen. Determination of an effective amount of an immunogenic or vaccine composition for administration to an organism is well within the capabilities of those skilled in the art.

[0059] A composition according to the invention may be for oral, systemic, parenteral, topical, mucosal, intramuscular, intravenous, intraperitoneal, intradermal, subcutaneous, intranasal, intravaginal, intrarectal, transdermal, sublingual, inhalation or aerosol administration. The composition may be arranged to be administered as a single dose or as part of a multiple dose schedule. Multiple doses may be administered as a primary immunization followed by one or more booster immunizations. The primary immunization may include a single formulation such as a virus (GC46) or DNA vaccine, followed by one or more booster immunizations with single or multiple formulations such as another virus (such as MVA) or recombinant protein. Suitable timings between priming and boosting immunizations can be routinely determined. A composition according to the present disclosure may be used in isolation, or it may be combined with one or more other immunogenic or vaccine compositions, and/or with one or more other therapeutic regimes.

[0060] The present disclosure thus provides a method of protecting a human or non-human mammal from the effects of malarial infection comprising administering to the human or non-human mammal a composition described herein. The composition may be a vaccine. The disclosure further provides a method for raising an immune response in a human or non-human mammal comprising administering a pharmaceutical composition described herein to the human or non-human mammal. The immune response is preferably protective. The method may raise a booster response in a patient that has already been primed. The immune response may be prophylactic or therapeutic.

EXAMPLES

Example 1: Identification of E140

[0061] A novel, highly protective pre-erythrocytic (PE) *Plasmodium* yoelii (Py) antigen, human orthologs for which are identified for use in a human malaria vaccine. This antigen is identified as PlasmoDB ID 10: PY06306, or PY17X_0210400, PYYM_0211900 or ID: 2121.m00052, depending on the nomenclature used. The antigen is also referred to as E140 or Py E140 in laboratory testing disclosed herein as a shorthand. The novel antigen is highly expressed in the sporozoite, liver, and blood stages of the parasite, and induces $CD8^+$ T cell responses in mice immunized with the *P.* yoelii radiation-attenuated sporozoites (RAS). It generates strong antibody and cellular responses upon antigen-specific vaccine immunizations and sterilely protects between 71% - 100% alone and in combination with other antigens of mice from an infectious *P. yoelii* sporozoite and blood stage challenges. First, *P.* yoelii pre-erythrocytic antigens were screened for their reactivity to T cells from RAS-immunized mice as a platform for identifying antigens for vaccine development. This process involved identifying, cloning, generating DNA plasmid (VR1020), screening, and evaluating Py antigens for ability to

protect mice. It is well recognized that mouse models are a predictor for success with human orthologs. The gene encoding the PY06306 antigen was identified as a pre-erythrocytic target for vaccine development, and the partial gene was cloned. Experiments then determined that the protein could recall cytokine (IFN-γ) responses from splenocytes generated in mice immunized with the *P. yoelii* RAS. This data provided strong evidence that the PY06306 antigen was involved in the RAS immune response and protection, therefore demonstrating pre-erythrocytic vaccine value in humans.

Example 2: Confirming E140 protection

[0062] Two vaccine reagents were made expressing the PY06306 antigen for protection studies in mice. These reagents were generated with the full-length gene: DNA vaccine in the VR1020 plasmid (PY06306-E140) and adenovirus serotype 5 (AdE1(t.PY06306)E3(10X)E4(TIS1)). The evidence for vaccine potential of the PY06306 antigen is shown in two separate animal matrix studies, intended to assess the ability of the antigen to induce an immune response capable of sterilely protecting mice from an infectious Py sporozoite challenge. The sterile protection was measured by the absence of parasites in the blood of mice examined up to 14 or 17 days post sporozoite challenge. Outbred CD1 mice were immunized with a regimen consisting of a prime with DNA vaccine (100 μg, IM) and a boost with adenovirus serotype 5 constructs (10^{10} PU, IM) 6 weeks later. A 3-antigen combination strategy (named matrix) was adopted to test the PY06306 antigen plus other new Py pre-erythrocytic antigens with and without *P.* yoelii circumsporozoite protein (PyCSP).

[0063] The first matrix animal study shown in Figure 1 revealed two PY06306-containing antigen combinations (groups) yielding significant protection. The first combination induced 64% and 86% sterile protection alone and with PyCSP, respectively. The antigen

components of this first combination were E140 (PY06306), E137 (PY05693) and E057 (PY03396). The 86% protection of the 3-antigen mixture combined with PyCSP was twice as high as the PyCSP alone group (43%), indicating a significant enhancement in the efficacy of this gold standard vaccine. The second 3-antigen combination produced 14% and 71% sterile protection alone and with PyCSP, respectively. This second combination consisted of E140 (PY06306) combined with two additional antigens with vaccine potential: Py325 (PY00232) and PyCeITOS (PY17X_1434600). Any or all of these five antigens (PY06306, PY05693, PY03396, PY325, and PyCeITOS) contributes to the protection shown in the corresponding figure; however, PY06306 was the only antigen common to all three antigen combinations, thus requiring a second experiment for the deconvolution of these antigen combinations.

Example 3: Sporozoite Challenge

[0064] A second study (Matrix Deconvolution Experiment 2) was designed to evaluate several antigen combinations having the PY06306 as the common denominator antigen. The experimental format and immunizations followed the same regimen as described for the first matrix experiment. Figures 1 and 2 show the markedly high efficacy for all antigen combinations that include the PY06306 (E140) antigen, ranging from 71% to 100% of the mice protected. Overall, 89% (137/154) of PY06306-immunized mice were protected from malaria infection. The PY06306 vaccine alone yielded 71% protection, significantly higher compared to 36% for the PyCSP alone group. Furthermore, there was a substantial delay in the onset of parasitaemia of non-protected mice as shown in figure 3. Detailed analysis of blood smears data from the PY06306-immunized group shows that three of the four non-protected mice became malaria positive on days 7, 10 and 12 after sporozoite challenge. This is significant when

compared to the parasitaemia onset of the PyCSP, 4X Null, and Naive groups, in which all nonprotected mice became malaria positive by day 5 post sporozoite challenge.

Example 4: Antibody Titers

[0065] The PY06306 antigen induces high antibody titers to *P.* yoelii sporozoite stages and low antibody levels to blood stages depending on the individual mouse. This evidence is shown in Figure 4 (PY06306 group) listing immune fluorescence (IFA) antibody titers to both sporozoite and blood stage parasites measured in pooled sera from mice in the Matrix Deconvolution Experiment 2. In summary, anti-sporozoite antibodies were detected in all groups immunized with PY06306, including combinations, which supports the immunogenicity of PY06306 antigen. Titers range from 1:5,120 to 1:20,480. Antibodies induced by the *P.* yoelii PY06306 immunization cross-reacts to *P.* berghei sporozoites. The detection of high antibody titers (1:5,120) in mice immunized with PY06306 alone demonstrates that the PY06306 antigen induces antibodies to sporozoites.

[0066] Two important observations based on a review of the data are: (i) the absence of protection (0%) and the lack of antibody response for the group of antigens without PY06306 (PY03396 and PY05693) in figure 2. This confirms that the PY06306 is the main, if not the only component of these combinations inducing protection. The other (ii) is the anti-sporozoite antibody response induced specifically by the PY06306 antigen. The comparison of the anti-sporozoite IFA titers for the protected versus non-protected mice strongly indicates that the antibodies detected in these mice correlate with the protection outcome. All protection studies were performed under animal protocols D02-09 and 14-IDD-13. The results of the protection studies validate the role of PY06306 orthologs as valuable components for a malaria vaccine.

Example 5: Spleen and Liver Analysis

[0067] Further studies confirmed that in spleen, > 10% CD8+ T cells expressing IFNγ and lower (< 0.6%) CD4+ T cells in PY06306-immunized mice. A range of 5% to 16.2% in liver was observed. High efficacy of protection continued 11 weeks after a second sporozoite challenge. The T cell depletion indicates that high levels of E140-specific T cells are not required for protection in mice. Additionally, PY06306 immunization induces high levels of CD8+ T cells expressing IFNγ in the spleen liver. Anti-PY06306 sera transfer to both CD1 and BALB/c mice significantly delayed the onset of parasitemia. E140-sera recipient mice also had significantly lower IFA titers compared to protected mice immunized with PY06306. PY06306 sera collected prior to sporozoite challenge reacts to sporozoites only. However, after challenge some protected mice developed antibodies positive to blood stage by IFA.

[0068] PY06306 sterilely protects up to 100% of CD1 and BALB/c mice from a blood stage challenge (figure 11). Immunization with PY06306 prevents blood infection and delays onset of detectable parasitemia in 88% (30/34) of non-protected mice. Additionally, transfer of anti- PY06306 antibodies to naïve mice significantly delays infection. High levels of CD8+ T cells expressing IFNγ in are found in spleens and livers of PY06306-immunized mice. Depletion did not reduce sterile protection. PY06306-specific IFA antibody titers correlate with protection.

Example 6: In vivo T cell depletion

[0069] Figure 9 shows the results of a study on in vivo t-cell depletion. Several groups of outbred CD1 mice were immunized. T cell depletions were performed by injection of T cell-specific monoclonal antibodies following standard protocols. Mice were then challenged with 300 *P. yoelii* sporozoites and protection assessed by the absence of parasites in thin blood smears up to 19 days after challenge. All PY06306-immunized mice that had their T cells depleted were protected, confirming that both CD4+ and CD8+ T cells are not required for the PY06306

protection. One non-protected mouse from the CD4/CD8 group had malaria detected in the blood 13 days post sporozoite challenge while all other mice had positive smears on day 5. A total of 68 protected mice out 70 were immunized, a 97% overall efficacy. This study confirmed the surprising mechanism that protection induced by a pre-erythrocytic antigen against a sporozoite challenge does not rely on T cells.

Example 7: Sera transfer studies

[0070] Figures 10A and 10B shows sera transfer studies in CD1 and BALB/c mice. This study confirmed the role of antibodies in the protection induced by PY06306 (E140). The study design followed standard sera transfer protocols, where sera from PY06306-immunized CD1 and BALB/c mice were harvested, transferred to naïve animals (1:1 ratio), and then challenged with *P. yoelii* sporozoites. Sera transfers took place over 2 days; 24 hours and 6 hours before the sporozoite challenge. The protection results are shown in Figure 10A for CD1 mice and Figure 10B for BALB/c mice. Figures 10A and 10B show that no sterile protection was transferred with sera (7% (1 out 14) of CD1 and 0% (0 out 14) of BALB/c) from mice immunized with PY06306 vaccine. There was a statistically significant delay in the onset of parasitemia on all non-protected mice from the PY06306 sera recipient (dotted line) as compared to any other group in the same study (Mantel-Cox ***, p=0.0001). This confirms that the anti-PY06306 antibodies have an effective impact on the parasite development in the blood play a role in the protection. Significantly lower antibody titers in the recipient CD1 (1:2,560) and BALB/c (1:575) mice and compared to the donor CD1 (1:7,994) and BALB/c (1:18:549) mice explain why these mice were not protected from the challenge.

Example 8: Detection of PY06306-specific CD8 T cells in Spleen and Liver

[0071] PY06306-specific CD8 T cells are found in the spleens and livers of PY06306-immunized and naïve mice. Due to the fact that PY06306 is a large molecule, 15mer overlapping peptides were divided into two pools spanning the entire protien; Pool A containing peptides from the N-terminal and Pool B from the C-terminal of PY06306. T cells were measured by flow cytometry gated for CD8+ cells expressing Interferon gamma (IFNγ) and expressed as a percentage of the total T cell population. The data shows that only peptides from Pool A were able to recall IFNγ CD8 cells confirming that PY06306 T cell epitopes are likely restricted to the N-terminal of the antigen. Very high levels of CD8+ T cells expressing IFNγ were detected for both spleens (average 18%) and livers (average 11%) of PY06306-immunized mice. For intracellular cytokine staining, splenocytes and liver-resident T cells were prepared from PY06306- and Null-immunized mice using standard protocols, followed by stimulation for six hours with a final concentration of 2μg/ml of PY06306 (E140) peptide pools A and B. Data were acquired using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star Inc.).

Example 9: PY06306 Induces Protection in BALB/c mice

[0072] PY06306 antigen effectively protects BAB/c strains of mice against a sporozoite challenge. Fourteen BALB/c mice per group were immunized with a dose of DNA and boosted with Adenovirus 5 encoding PY06306, PY06306 + PyCSP, and PyCS*P*. Null-immunized and naïve were used as negative control groups of mice. All mice were challenged with 100 infectious *P. yoelii* sporozoites and parasitaemia monitored for 17 days after challenge by Giemsa-stained thin smears. Upon challenge all (100%) PY06306-immunized mice were sterilely protected (PY06306 and PY06306+PyCSP) whereas 57% of PyCSP were protected.

Thus PY06306 can protect an inbred strain of mice, and mixing with PyCSP antigen does not inhibit the PY06306 protection.

Example 10: PY06306 Induces Protection against a Blood Stage Challenge

[0073] Figure 11 shows PY06306 protection against a blood stage challenge. PY06306 antigen alone and in combination with PyFalstatin protects mice against a stringent challenge with 10,000 blood stage parasites. In this study, mice immunized with PY06306 alone and in combination with PyFalstatin and challenged with *P. yoelii*-infected erythrocytes. Both groups of mice were 100% sterilely protected (black and gray bars). PyFalstatin antigen is also known as PY03424. The protection against a blood stage challenge provides a second level of defense induced by the PY06306 vaccine, a valuable feature for a malaria vaccine.

Example 11: Protection with Lower and Single dose of Codon-optimized PY06306 Ad5

[0074] Figure 12 shows protection with codon-optimized Adenovirus 5. This study evaluated an Adenovirus 5 construct made with codon-optimized (co) PY06306 gene designed for expression in mammalian cells. The change in the PY06306 native codon sequence did not alter the amino acid sequence expressed by the Ad5 virus. A study examined and compared the *in vitro* expression of the PY06306 protein expressed by the native (na) and codon-optimized (co) Adenovirus 5 constructs. After probing with mouse polyclonal sera, the coPY06306 Ad5 expresses much higher levels of PY06306 protein compared to the native construct. In the first groups of mice, the boosting dose was titrated for both native (na) and codon-optimized PY06306 Ad5 ranging from 10^10, 10^9, 10^8 and 10^7 PU per dose. All mice in these eight groups were primed with the same coPY06306 DNA vaccine dose (100 μg) and boosted with varying doses of either naPY06306 (black bars) or coPY06306 (gray bars) Ad5 construct intramuscular (IM). The overall efficacy indicates that the co PY06306 Ad5 vaccine induces

higher protection in CD1 mice (100%, 100%, 86% and 93%) compared to the na PY06306 had lower protection (86%, 93%, 86% and 71%) for the same Ad5 doses. The study also compared subcutaneous (SC) and intravenous (IV) routes for Ad5 administration. SC route yielded similar protection levels for both na and co PY06306 vaccine (50 and 57% respectively). The IV route for the co PY06306 Ad5 resulted in 100% sterile protection, while the na provided 79%. The IV route for na PY06306 Ad5 yielded 79% sterile protection, while subcutaneous yielded 50% protection. Mice groups immunized with a single dose of coPY06306 Ad5 induced 93% sterile protection compared to 29% for the naPY06306 vaccine. These mice received no DNA vaccine priming. All protection studies were performed under animal protocols D02-09 and 14-IDD-13.

Example 12: Human *P. falciparum* is immunogenic in mice

[0075] Figure 13 shows that *P. falciparum* PFA0205w (E140 ortholog) is immunogenic in mice. Four vaccine reagents were generated for PFA0205w (aka PF3D7_0104100), these are: VR1020 DNA vaccine construct, human Adenovirus 5 construct, protein expression plasmids pEU-E01-GST, and pEU-E01-His. DNA vaccine and Ad5 were produced in large scale for mice immunizations. Recombinant proteins were produced in small-scale by the wheat germ cell-free system at NMRC. Both CD1 and BALB/c mice were immunized using a variety of prime-boost regimens as shown in figure 13. The Ad5 prime and recombinant protein boost was the most immunogenic regimen, inducing IFA titers up to 1:4,000 to both *P. falciparum* blood and sporozoite stage parasites. A single dose of PFA0205w Adeovirus 5 induce antibodies to parasites. This confirms that PFA0205w as a single dose of recombinant virus (Adenovirus 5) or as a prime-boost with an Ad5-protein regimen are viable as vaccine formulations.

Example 13: *P. falciparum* E140 (PFA0205w) is immunogenic in humans

[0076] Figure 14 shows that the *P. falciparum* E140 (PFA0205w) is immunogenic in humans. T cells from individual subjects immunized with radiation-attenuated sporozoites (RAS) were able to respond to stimulation with PFA0205w peptide pool (A). The peptide mixture contained 15mer overlapping peptides covering most of the N-terminus region of PFA0205w protein. Since the protein is large, the peptides were divided into two pools; pool A covering the N-terminus and pool B for the C-terminus of the PFA0205w protein. The data in both graphs indicated that the imuunizations with the attenuated sporozoite vaccine induce both CD4 and CD8 T cells in humans. CD4+ and CD8+ T cells play a role in the PFA0205w-induced protection against pre-erythrocytic parasites. There are high levels of *P. yoelii* E140 responses in the spleen and livers of E140-immunized mice.

Example 14: PFA0205w is expressed in Schizonts and Localized in the Surface and Cytosol of Sporozoites

[0077] The PFA0205w antigen is expressed at both the sporozoite and schizont stages of *P. falciparum*. The IFA reactivity was obtained using CD1 mice serum generated by priming with PFA0205w Adenovirus 5 and boosting with recombinant PFA0205w protein. The serum was positive for 36-hour *P. falciparum* erythrocytic schizonts and negative for early rings and trophozoites. The subcellular localization of PFA0205w antigen in sporozoites was determined by immuno electron microscopy (EM). The analysis of micrographs showed that PFA0205w antigen is localized in both at the surface and in the cytosol of *P. falciparum* sporozoites. Immuno fluorescence and immuno electron microscopy showed reactivity of serum from CD1 mice immunized with PFA0205w Adeno 5 and boosted with recombinant PFA0205w protein. Air-dried IFA slides were made with NF54 *P. falciparum* parasites about 36 hours after invasion of red blood cell. IFA was performed with 1:500 serum dilution and developed with a FITC-

labeled goat anti-mouse Ig. For immuno EM, *P. falciparum* sporozoites-containing salivary glands were isolated from infected mosquitoes. Fixed glands were embedded, sectioned, mounted on electron microscopy grids and stained using same serum and colloidal gold-labeled anti-mouse antibodies. Micrographs confirmed that the PFA0205w antigen is localized in both at the surface and in the cytosol of *P. falciparum* sporozoites.

Example 15: PVX_081555 (PvE140) is expressed in *P. vivax* sporozoites

[0078] Figure 15 shows that PVX_081555 (PvE140) is expressed in *P. vivax* sporozoites. *Anopheles dirus* mosquitos were fed blood through a membrane feeder from patients infected with *P. vivax* malaria. Fourteen days after the membrane feeding the mosquito salivary glands were extracted from 100 mosquitos. The salivary glands were crushed in a microcentrifuge tube containing phosphate-buffered-saline with a pestle and to liberate *Plasmodium vivax* sporozoites. The salivary gland debris-sporozoite mixture was then centrifuged to remove the mosquito salivary gland debris and the *P. vivax* sporozoites were transferred from the supernatant to a new microcentrifuge tube. The extracted *P. vivax* sporozoites were counted and 1X106 sporozoites were digested with 1 ug of molecular biology grade trypsin at 37 degrees Celsius for 18 hours. After digestion the trypstic sporozoite peptides were desalted over a C8 reversed phase column and lyophilized. The lyophilized tryptic peptides were subjected to multi-dimensional-protein-identification technology (MudPIT) to identify *P. vivax* sporozoite proteins that might be vaccine candidates. Tandem mass spectra generated from *P. vivax* sporozoites were searched against a combined Anopheles-*Plasmodium vivax* protein sequence database using the Sequest algorithm. Output files from the Sequest search were loaded into the Scaffold protein viewer. Sequences matching the Anopheles proteome were subtracted using the Scaffold program to highlight proteins that specifically matched the *P. vivax* proteome. Scaffold software was used to compare

the abundance of each of the *P. vivax* sporozoite proteins identified by MudPIT. Protein abundance was defined by the Scaffold "quantitative value" which normalizes the abundance of mass spectra matching a given protein to that protein's molecular weight. 256 high-confidence *P. vivax* proteins were identified in this MudPIT experiment. *P. vivax* E140 (PVX_081555) was the 39th most abundant *P. vivax* sporozoite protein and the 5th most abundant membrane-associated protein sequenced. In comparison, the CSP vaccine antigen, that is also associated with the parasite membrane, was the 5th most abundant protein overall and the most abundant membrane-associated protein in the sample. This result illustrates that *P. vivax* E140 is among the most abundant membrane-associated proteins in the parasite. E140's membrane association and abundance therefore make it an exceptional target of the humoral response.

CLAIMS

I claim:

1. An immunogenic composition for protecting a mammal against malaria, the immunogenic composition comprising

a recombinant polypeptide,

wherein the recombinant polypeptide comprises one of the amino acid sequence of SEQ ID NO. 3, SEQ ID NO. 6, and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with one of SEQ ID NO. 3 and SEQ ID NO. 6;

a pharmaceutically acceptable carrier; and

an adjuvant.

2. An immunogenic composition for protecting a mammal against malaria, the immunogenic composition comprising

a combination of two or more recombinant polypeptides in a pharmaceutically acceptable carrier,

wherein a first one of the two or more recombinant polypeptides comprises one of the amino acid sequence of SEQ ID NO. 3 and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with SEQ ID NO. 3;

a pharmaceutically acceptable carrier; and

an adjuvant.

3. The immunogenic composition of claim 2 wherein a second one of the two or more recombinant polypeptides comprises an amino acid sequence selected from the group consisting of SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with one of SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, and SEQ ID NO. 9.

4. A method of inducing an immune response against malaria in a mammal, which method comprises

administering to said mammal an immunologically effective amount of a composition comprising a polypeptide encoded by one of the amino acid sequence of SEQ ID NO. 3, SEQ ID NO 6, and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with one of SEQ ID NO. 3, and SEQ ID NO. 6.

5. The method of claim 4 wherein the mammal is a human.

6. The method of claim 4 wherein the method further comprises administering to a mammal one or more priming or boosting immunizations against malaria, wherein said priming and boosting immunizations comprise an immunologically effective amount of a recombinant polypeptide, wherein the recombinant polypeptide comprises one of the amino acid sequence of SEQ ID NO. 3, SEQ ID NO. 6 and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with one of SEQ ID NO. 3, and SEQ ID NO. 6.

7. A method of administering to a mammal an immunologically effective amount of the composition of claim 1 by introducing into the mammal a suitable expression vector for expressing the polypeptide, wherein the suitable expression vector is selected from the group consisting of a plasmid, replicating viral vector, and nonreplicating viral vector.

8. The method of claim 7 wherein the mammal is a human.

9. The immunogenic composition of claim 1 wherein the recombinant polypeptide is expressed by a suitable expression vector selected from the group consisting of a plasmid, replicating viral vector, and nonreplicating viral vector.

10. The immunogenic composition of claim 1 wherein the recombinant polypeptide is expressed by a suitable expression vector selected from the group consisting of a DNA plasmid,

baculovirus, VSV, MVA, GC46, alphavirus replicon, adenovirus, poxvirus, adenoassociated virus, cytomegalovirus, canine distemper virus, yellow fever virus, retrovirus, RNA replicons, DNA replicons, alphavirus replicon particles, Venezuelan Equine Encephalitis virus, Semliki Forest Virus, and Sindbis Virus.

11. The method of claim 4 the composition is administered through a suitable expression vector expressing the recombinant polypeptide, wherein the suitable expression vector is selected from the group consisting of a DNA plasmid, baculovirus, VSV, MVA, GC46, SpyVLPs, alphavirus replicon, adenovirus, poxvirus, adenoassociated virus, cytomegalovirus, canine distemper virus, yellow fever virus, retrovirus, RNA replicons, DNA replicons, alphavirus replicon particles, Venezuelan Equine Encephalitis virus, Semliki Forest Virus, and Sindbis Virus.

12. An immunogenic composition for protecting a mammal against malaria, the immunogenic composition comprising

an recombinant polypeptide,

wherein the recombinant polypeptide comprises one of the amino acid sequence of SEQ ID NO. 3, SEQ ID NO. 6, and derivatives thereof, wherein said derivatives have at least 10 contiguous amino acids of and/or 85% identity with one of SEQ ID NO. 3 and SEQ ID NO. 6;

wherein the immunogenic composition is a dry powder.

13. The immunogenic composition of claim 12 wherein the dry powder is suitable for administration to a mammal upon suspension or reconstitution in a pharmaceutically acceptable carrier.

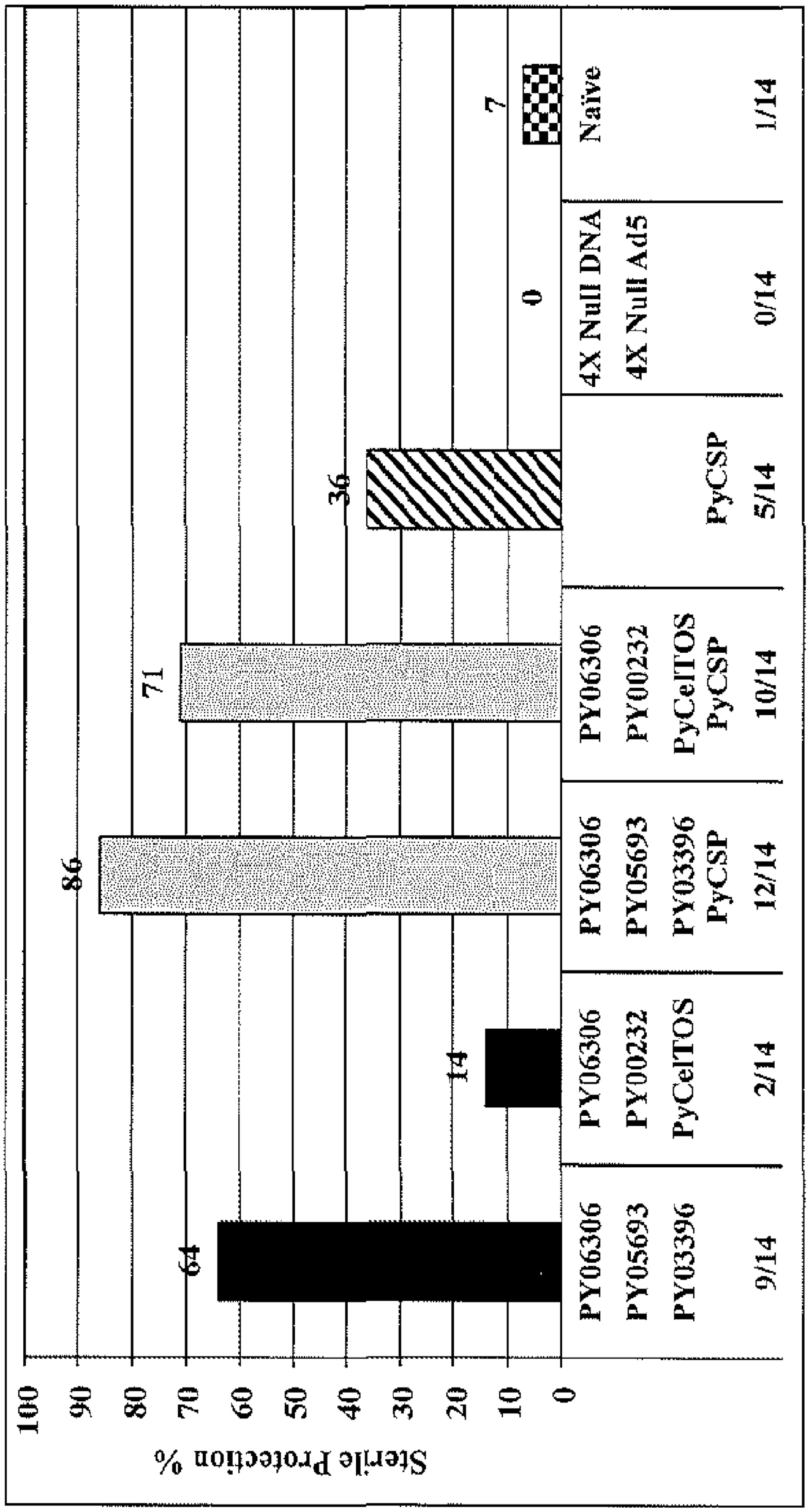
Sterile Protection %
100
90
80
70
60
50
40
30
20
10
0
64
9/14
PY06306
PY05693
PY03396
14
2/14
PY06306
PY00232
PyCelTOS
86
12/14
PY06306
PY05693
PY03396
PyCSP
71
10/14
PY06306
PY00232
PyCelTOS
PyCSP
36
5/14
PyCSP
0
0/14
4X Null DNA
4X Null Ad5
7
1/14
Naïve

FIG. 1

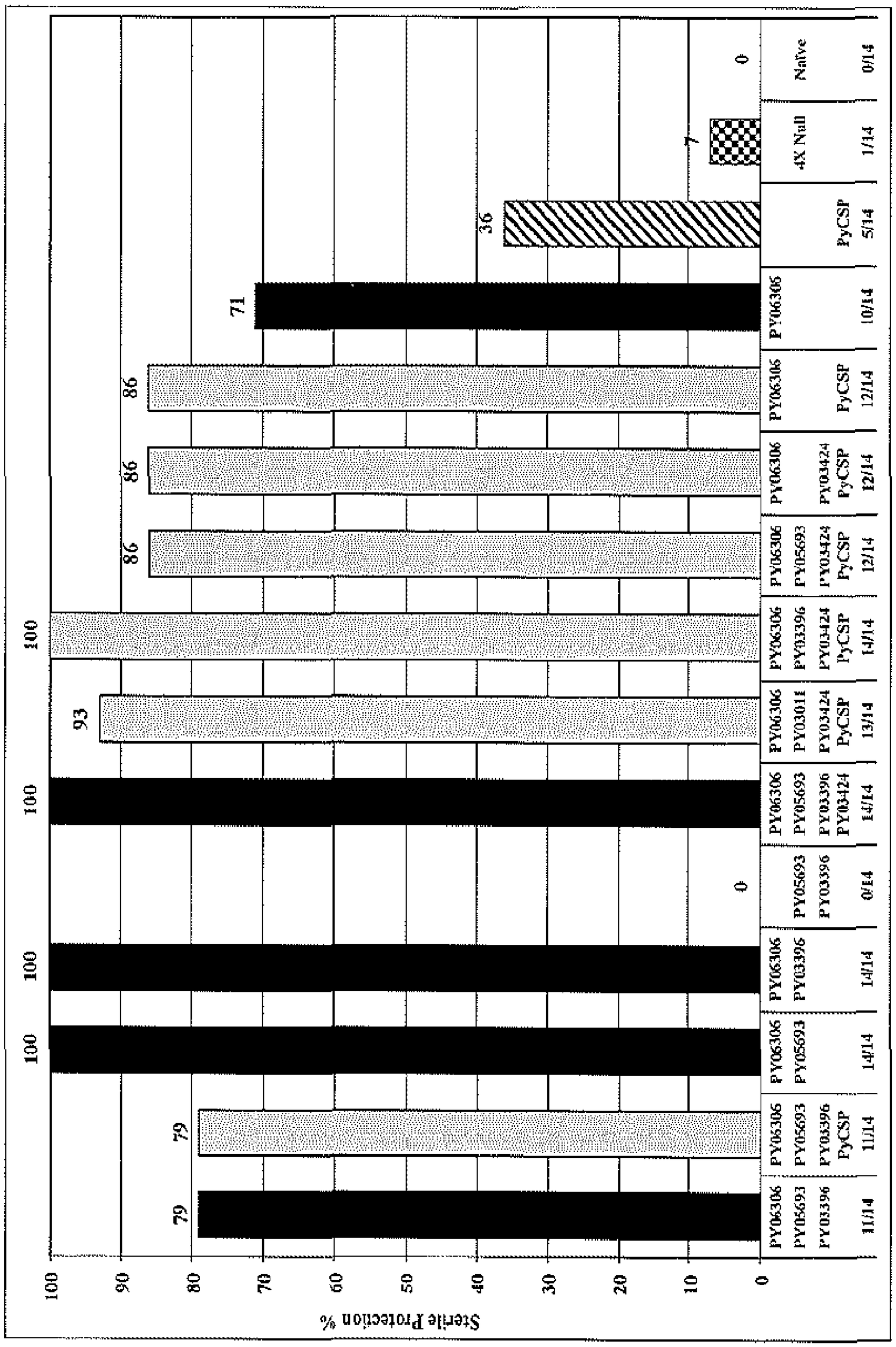
Sterile Protection %
100
90
80
70
60
50
40
30
20
10
0
93
100
100
100
79
86
86
86
71
36
0
PY06306 PY03011 PY03424 PyCSP 13/14
PY06306 PY03396 PY03424 PyCSP 14/14
PY06306 PY05693 PY03424 PyCSP 12/14
PY06306 PY03396 PY03424 PyCSP 12/14
PY06306 PyCSP 12/14
PY06305 10/14
PyCSP 5/14
Native 0/14
4X Null 1/14
PY06306 PY05693 PY03396 PY03424 14/14
PY05693 PY03396 0/14
PY06306 PY05693 PY03396 PY03424 14/14
PY06306 PY05693 14/14
PY06306 PY05693 PY03396 PyCSP 11/14

FIG. 2

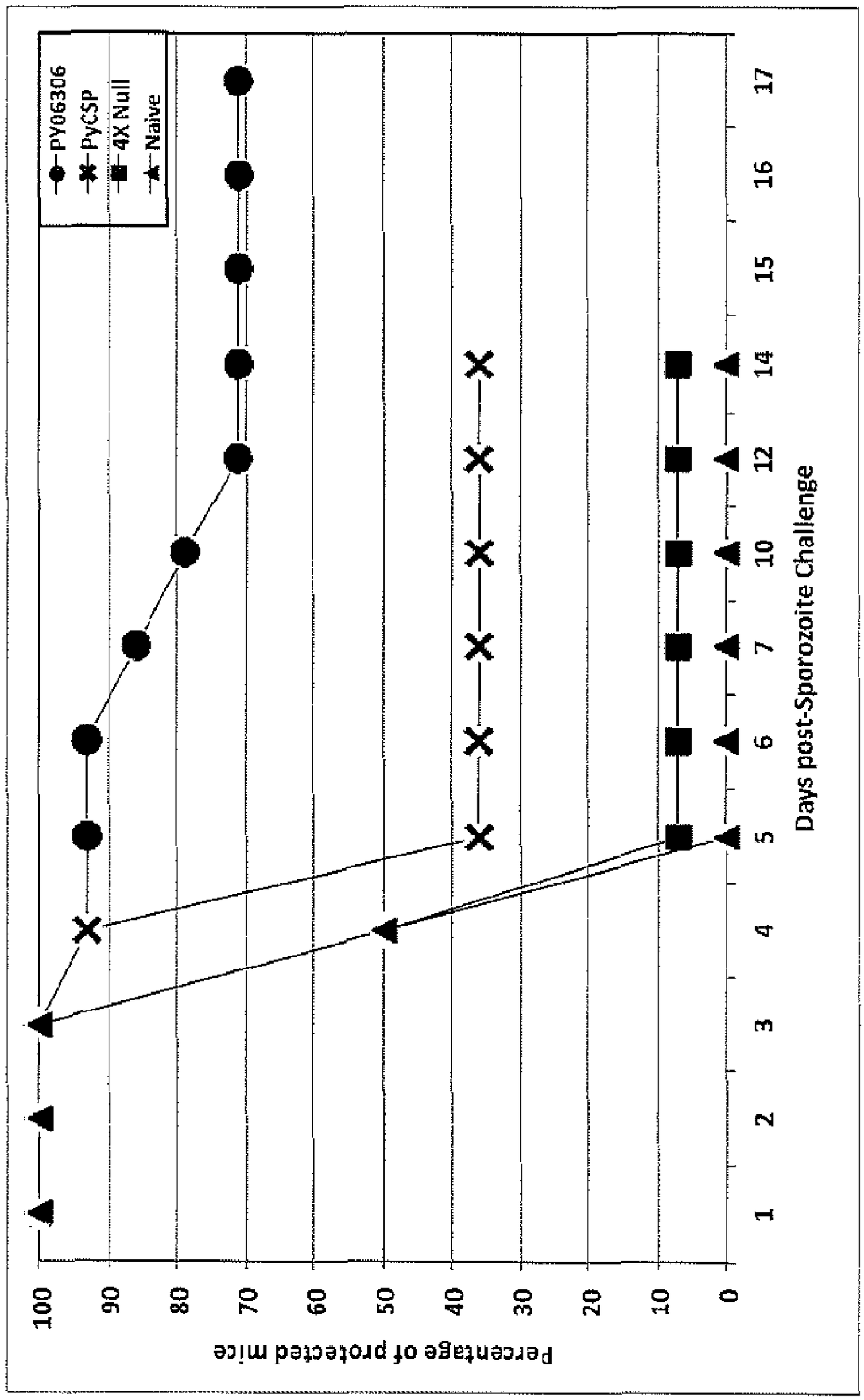
PY06306
PyCSP
4X Null
Naive
Days post-Sporozoite Challenge
1
2
3
4
5
6
7
10
12
14
15
16
17
Percentage of protected mice
100
90
80
70
60
50
40
30
20
10
0

FIG. 3

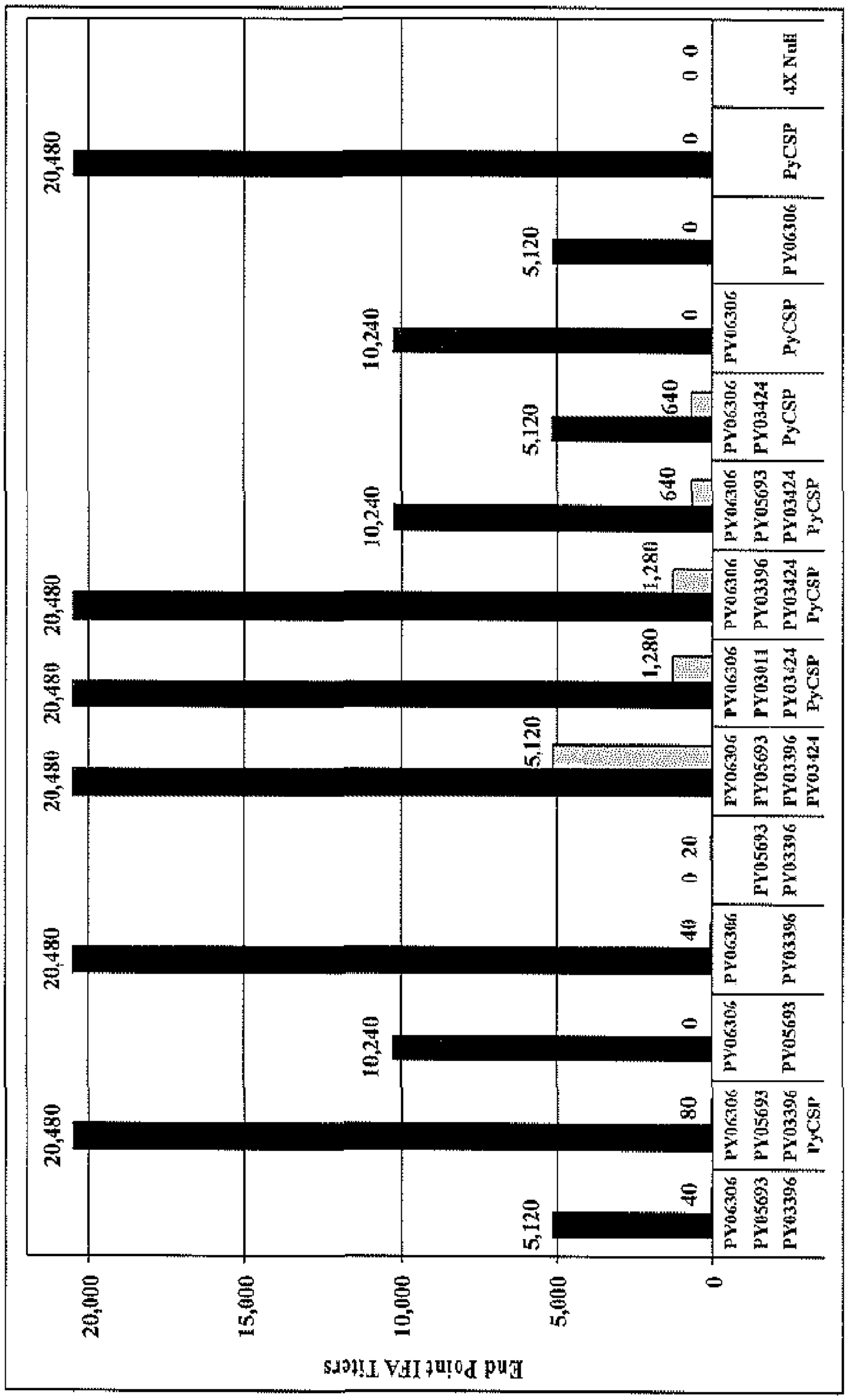
End Point IFA Titers
20,000
15,000
10,000
5,000
0
4X NuH
PyCSP
PY06306
20,480
10,240
5,120
640
1,280
40
80
0
20
PY06306
PY03396
PY03424
PyCSP
PY05693
PY03011

FIG. 4

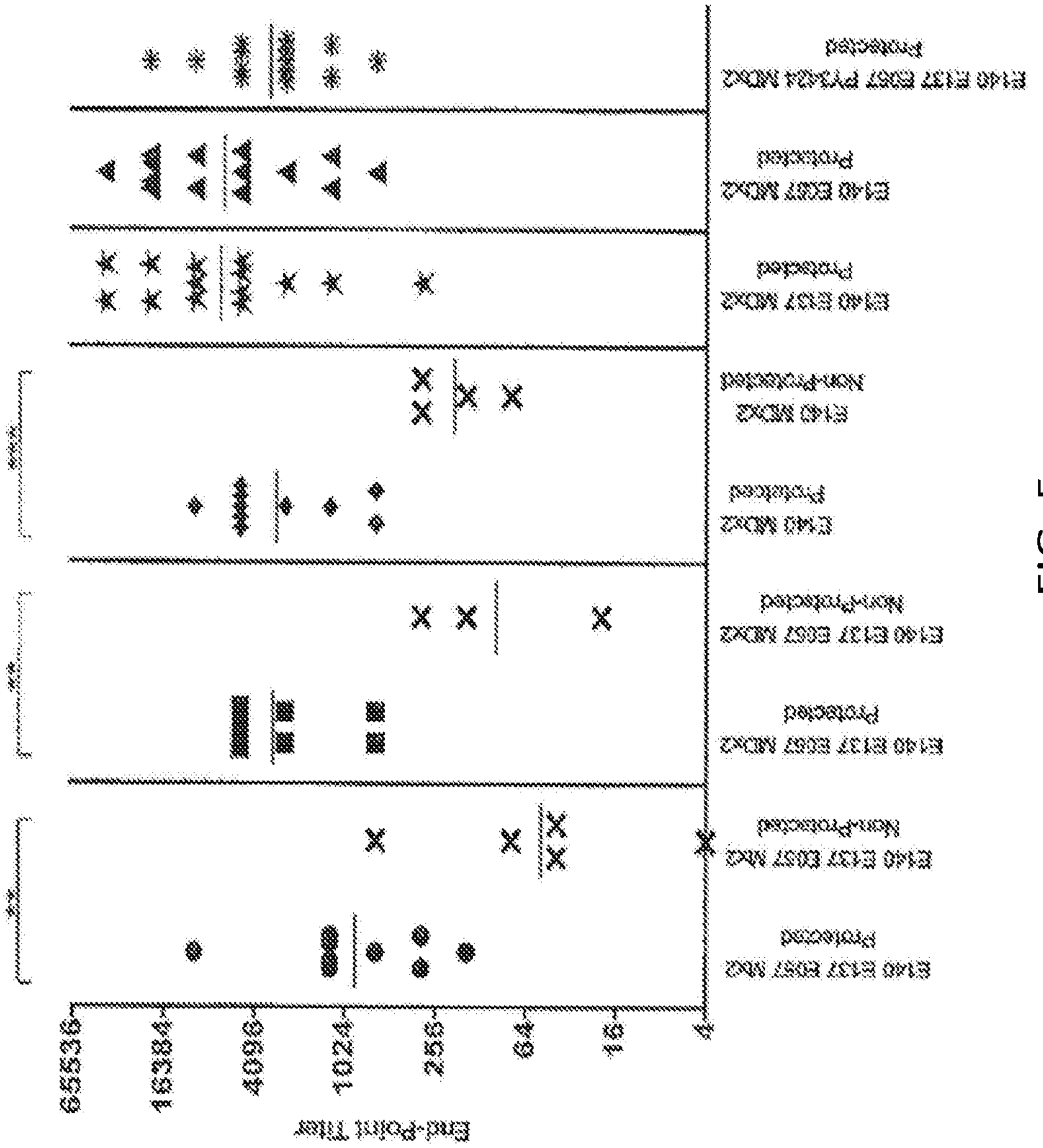
End-Point Titer
65536
16384
4096
1024
256
64
16
4
E140 E137 E057 NO2
Protected
E140 E137 E057 NO2
Non-Protected
E140 E137 E067 NO2
Protected
E140 E137 E067 NO2
Non-Protected
E140 E137 E067 NO2
Protected
E140 E137 E067 NO2
Non-Protected
E140 NO2
Protected
E140 NO2
Non-Protected

FIG. 5

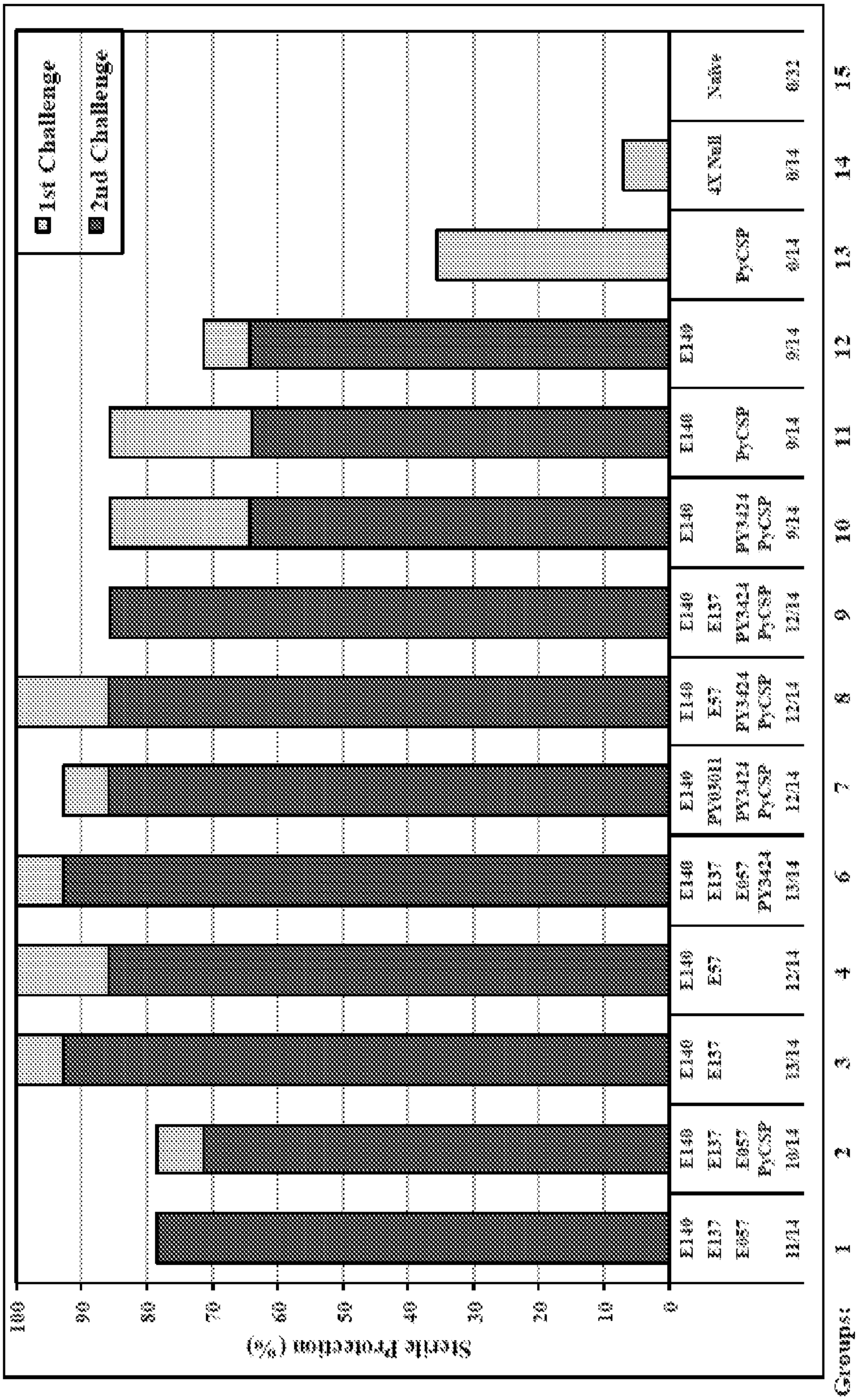
E140 Induces Long-Lasting Protection (11 Weeks)
Deconvolution Study – Combined Protection from 2 Challenges
1st Challenge
2nd Challenge
Sterile Protection (%)
100
90
80
70
60
50
40
30
20
10
0
Groups:
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
E140
E137
E057
H14
E140 PY03011 PY3424 PyCSP 12/14
E140 E137 E057 PY3424 10/14
E140 E137 PY3424 PyCSP 13/14
E140 E137 PY3424 PyCSP 12/14
E140 E148 PY3424 PyCSP 9/14
E140 E148 PyCSP 9/14
E140 E148 9/14
E140 E148 E137 E057 PY3424 13/14
E140 E148 E137 E57 12/14
E140 E148 PY3424 PyCSP 9/14
E140 E148 PyCSP 9/14
E140 E148 9/14
E140 PyCSP 8/14
4X Null 8/14
Naïve 6/32

FIG. 6

CA 03014196 2018-08-09

E140 Antigen Homology Amongst *Plasmodium spp*

	Pf E140	Pv E140	Py E140	Pb E140	Pc E140	Pk E140	Pr E140	Pg E140
Pf E140	---							
Pv E140	43%	---						
Py E140	35%	37%	---					
Pb E140	36%	36%	86%	---				
Pc E140	37%	37%	67%	67%	---			
Pk E140	43%	78%	36%	36%	34%	---		
Pr E140	92%	43%	35%	35%	37%	43%	---	
Pg E140	81%	44%	34%	35%	35%	43%	82%	---

Pf - *P. falciparum*	Pc - *P. chabaudi*	Pk - *P. knowlesi*
Pv - *P. vivax*	Py - *P. yoelii*	Pr - *P. reichenowi*
	Pb - *P. berghei*	Pg - *P. gaboni*

FIG. 7

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E140 Antigen is Highly Conserved Amongst *P. falciparum* Strains

	3D7	UGT5.1	7G8	Mali	UGPA	HB3	Santa Lucia	IGH-CR14	FCH/4	NF135/5.C10	Tanzania	FVO
3D7*	—											
UGT5.1**	98%	—										
7G8 (Brazil)	97%	97%	—									
Mali PS096	97%	99%	96%	—								
UGPA***	97%	97%	97%	98%	—							
HB3 (Honduras)	97%	97%	98%	98%	98%	—						
Santa Lucia****	97%	97%	97%	98%	98%	98%	—					
IGH-CR14§	97%	96%	96%	97%	97%	97%	97%	—				
FCH/4※	97%	98%	97%	98%	97%	98%	99%	96%	—			
NF135/5❖	97%	98%	97%	99%	98%	98%	99%	96%	99%	—		
Tanzania	93%	93%	94%	92%	93%	93%	93%	92%	93%	93%	—	
FVO (Vietnam)	96%	96%	95%	97%	96%	96%	98%	95%	98%	98%	95%	—
Dd2 (Indochina)	97%	96%	97%	94%	96%	96%	96%	94%	96%	96%	98%	96%

* - The Netherland
** - Uganda
*** - Uganda/palo Alto
**** - El Salvador

§ - India (Rourkela, Orissa)
※ - The Philippines
❖ - Cambodia (CHMI)

FIG. 8

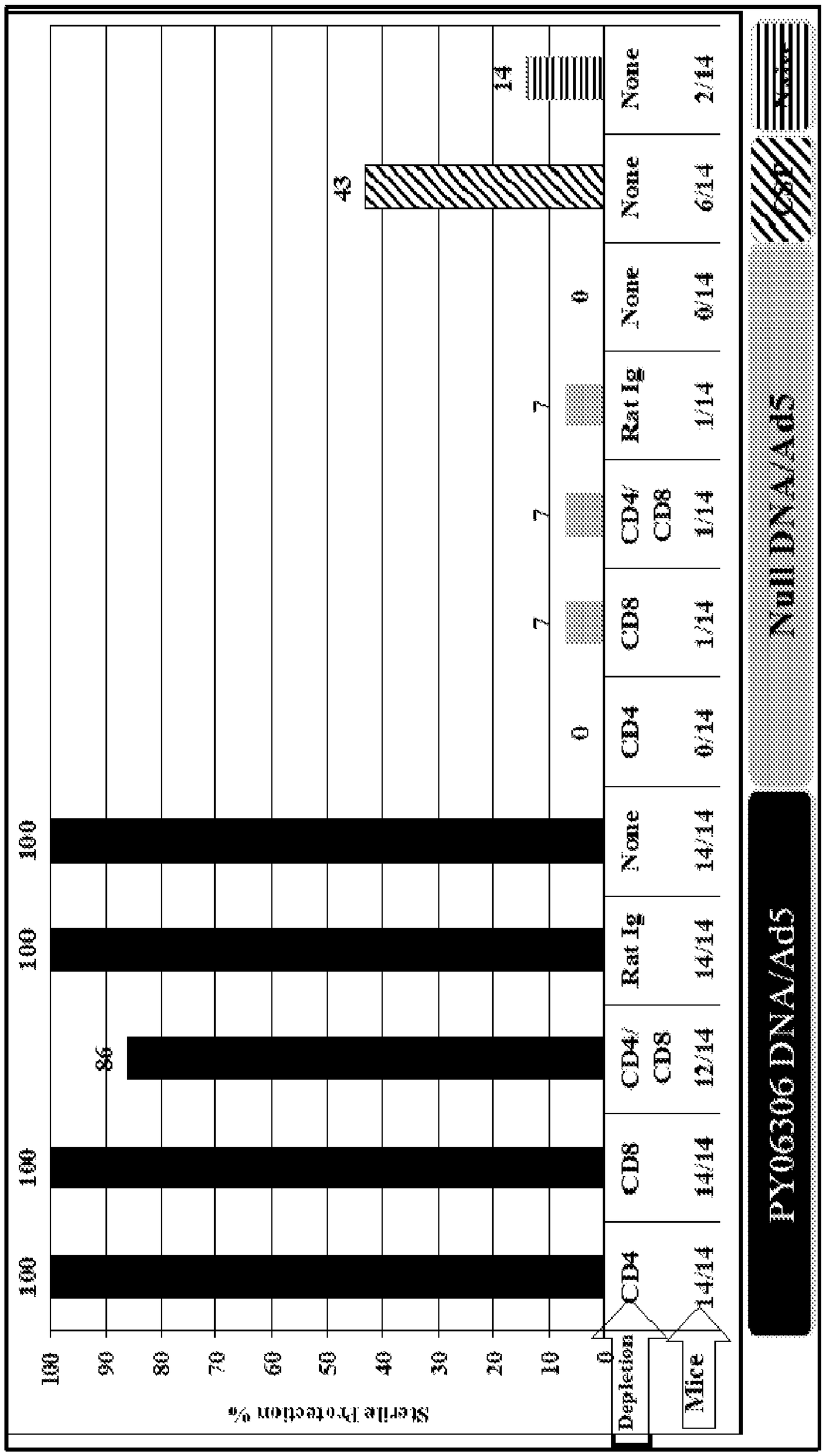
In vivo T cell depletion.
Sterile Protection %
100
90
80
70
60
50
40
30
20
10
0
Depletion
Mice
CD4
14/14
100
86
100
100
0
CD8 14/14
CD4/CD8 12/14
Rat Ig 14/14
None 14/14
CD4 0/14
PY06306 DNA/Ad5
7
7
7
0
43
CD8 1/14
CD4/CD8 1/14
Rat Ig 1/14
None 0/14
None 6/14
PY06306 DNA/Ad5
14
None
2/14
None
CD8

FIG. 9

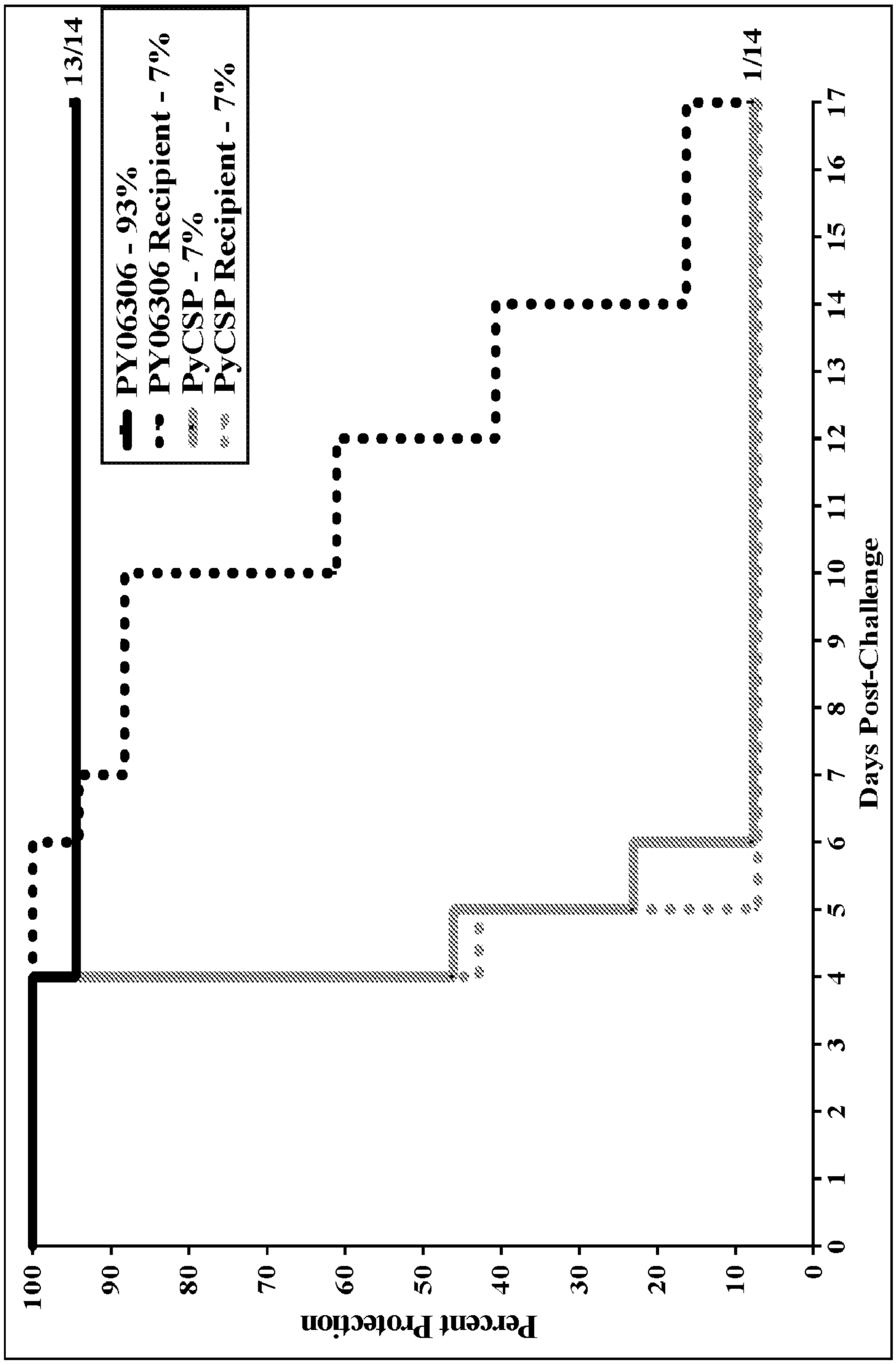
PY06306 - 93%
PY06306 Recipient - 7%
PyCSP - 7%
PyCSP Recipient - 7%
13/14
1/14
Percent Protection
Days Post-Challenge
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

FIG. 10A

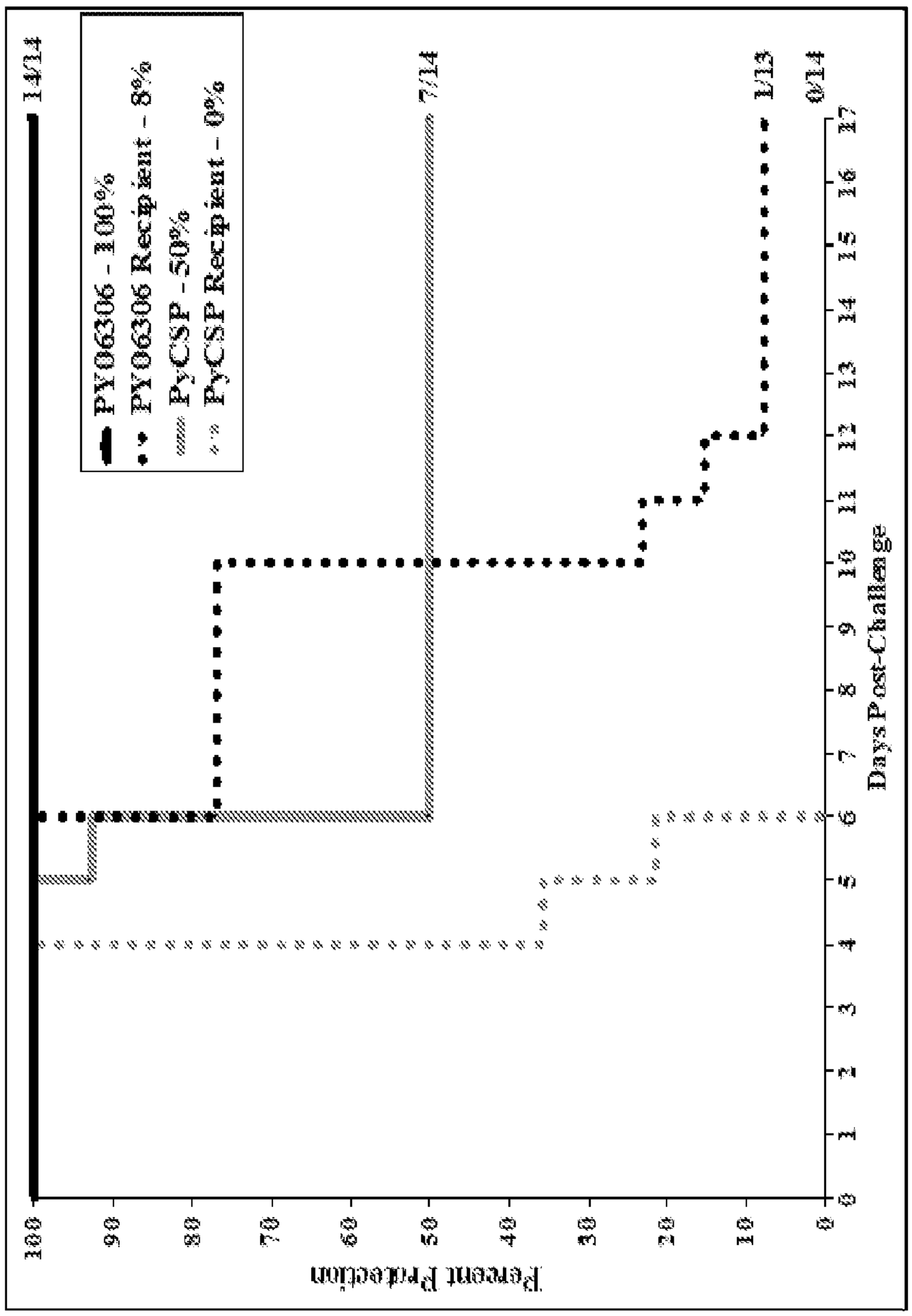
PY06306 - 100%
PY06306 Recipient - 8%
PyCSP - 50%
PyCSP Recipient - 0%
14/14
7/14
1/13
0/14
Percent Protection
Days Post-Challenge

FIG. 10B

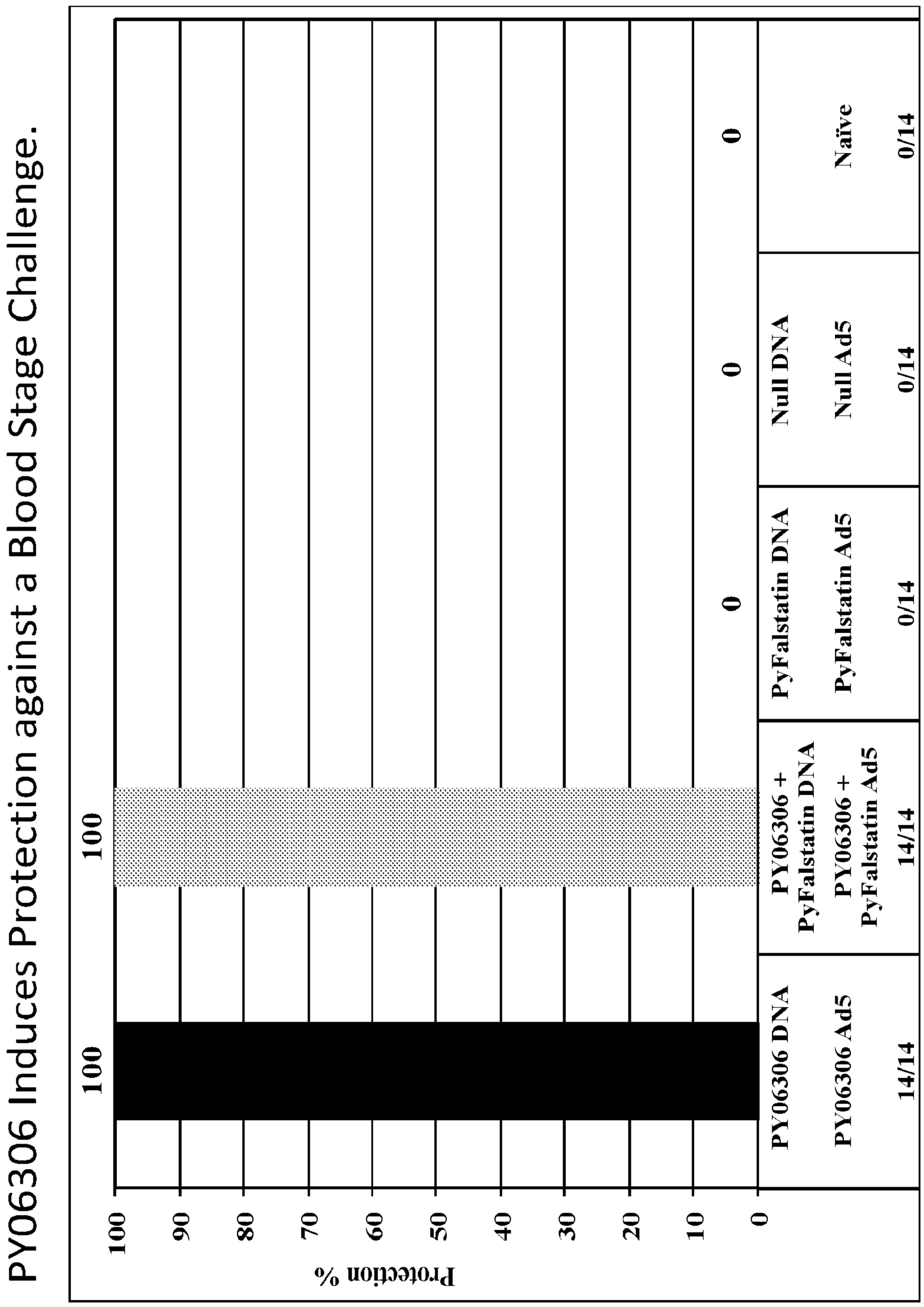
PY06306 Induces Protection against a Blood Stage Challenge.
Protection %
100
90
80
70
60
50
40
30
20
10
0
100
0
0
0
PY06306 DNA
PY06306 Ad5
14/14
PyFalstatin DNA
PyFalstatin Ad5
0/14
Null DNA
Null Ad5
0/14
PY06306 + PyFalstatin DNA
PY06306 + PyFalstatin Ad5
14/14
Naïve
0/14

FIG. 11

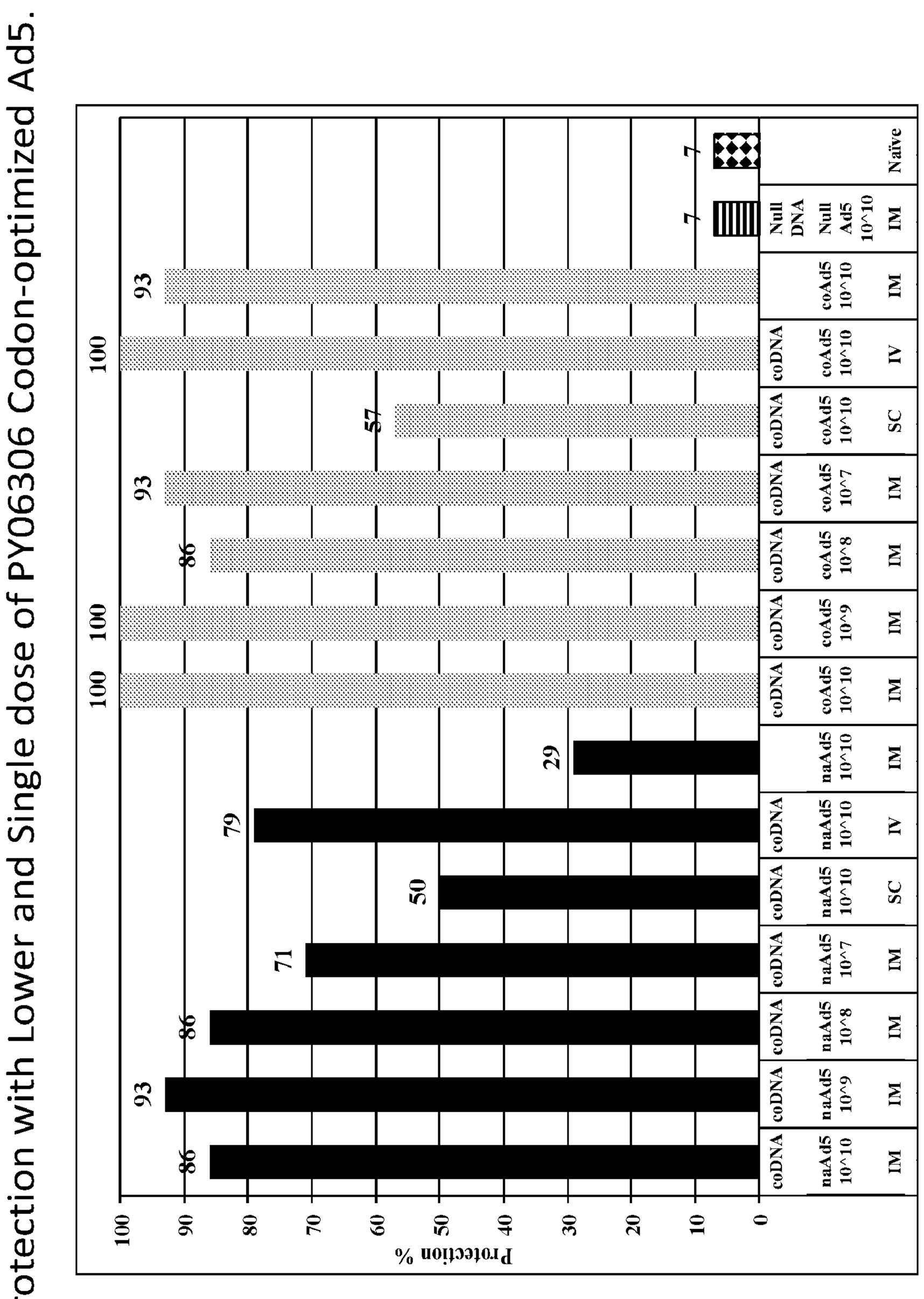
Protection with Lower and Single dose of PY06306 Codon-optimized Ad5.
Protection %
100
90
80
70
60
50
40
30
20
10
0
93
86
71
79
50
29
100
93
86
57
100
93
7
7
coDNA naAd5 10^10 IM
coDNA naAd5 10^9 IM
coDNA naAd5 10^8 IM
coDNA naAd5 10^7 IM
coDNA naAd5 10^10 SC
coDNA naAd5 10^10 IV
naAd5 10^10 IM
coDNA coAd5 10^9 IM
coDNA coAd5 10^8 IM
coDNA coAd5 10^7 IM
coDNA coAd5 10^10 SC
coDNA coAd5 10^10 IV
coAd5 10^10 IM
coDNA coAd5 10^10 IM
Null DNA IM
Null Ad5 10^10 IM
Naïve

FIG. 12

P. falciparum PFA0205w (PfE140) is Immunogenic in Mice.

	CD1 IFA Titer		BALB/c IFA Titer	
Vaccines	**Sporozoite**	Blood Stage	**Sporozoite**	Blood Stage
PFA0205w DNA/Ad5	**Negative**	Negative	**250**	Negative
PFA0205wDNA/Ad5	**20**	Negative	**250**	Negative
PFA0205w Ad5/GST Protein	**1000**	500	**1000**	250
PFA0205w Ad5/GST Protein	**2000**	4000	**500**	Negative
PFA0205w DNA x3	**Negative**	Negative	**250**	Negative
PFA0205w DNA x3	**Negative**	Negative	**250**	Neat
PFA0205w His Protein	**Negative**	Negative	**Neat**	Negative
PFA0205w His Protein	**Negative**	Negative	**Neat**	Negative
PFA0205w GST Protein	**Negative**	20	**250**	20
PFA0205w GST Protein	**Negative**	20	**Negative**	250

FIG. 13

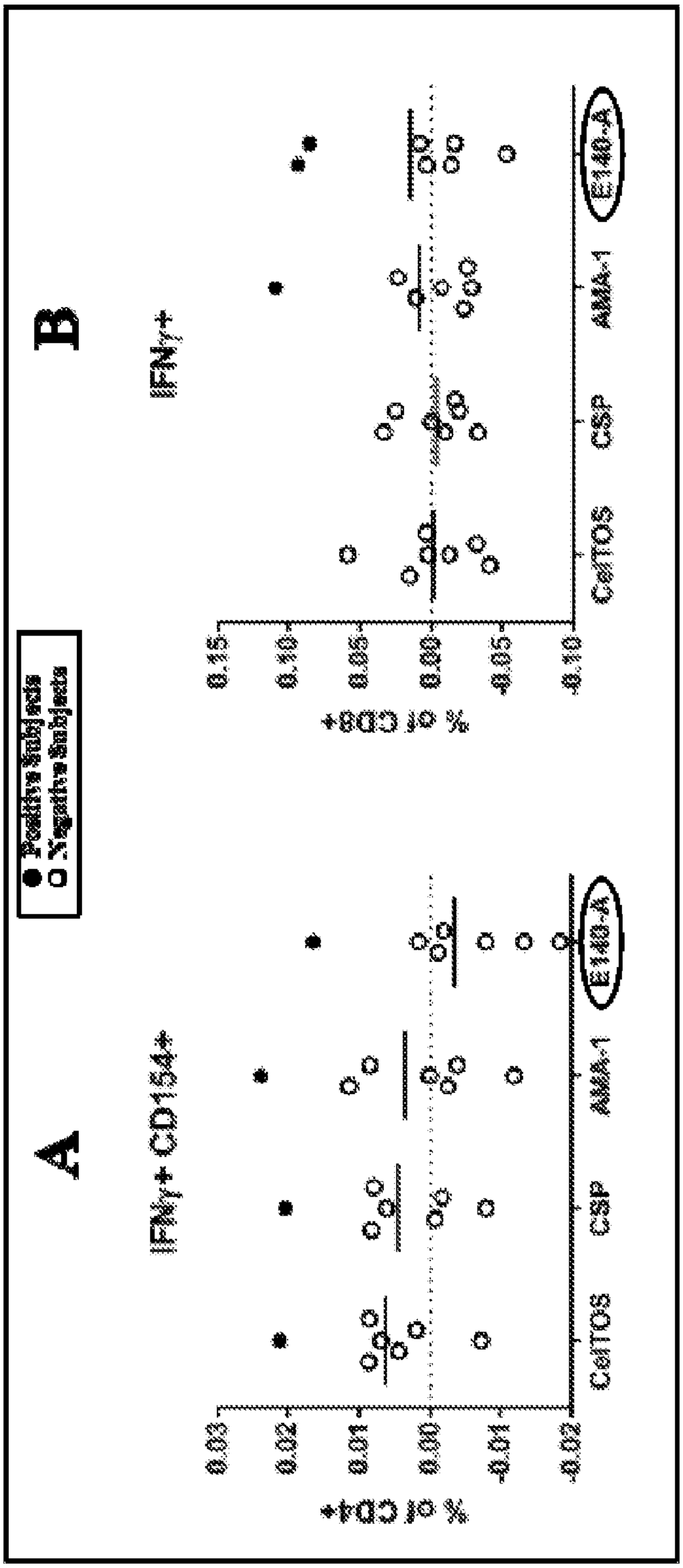
A
Pathogenic Subjects
Xenopathogenic Subjects
IFNγ+ CD154+
% of CD4+
0.03
0.02
0.01
0.00
-0.01
-0.02
CelTOS
CSP
AMA-1
E140-A
B
IFNγ+
% of CD8+
0.15
0.10
0.05
0.00
-0.05
-0.10
CelTOS
CSP
AMA-1
E140-A

FIG. 14

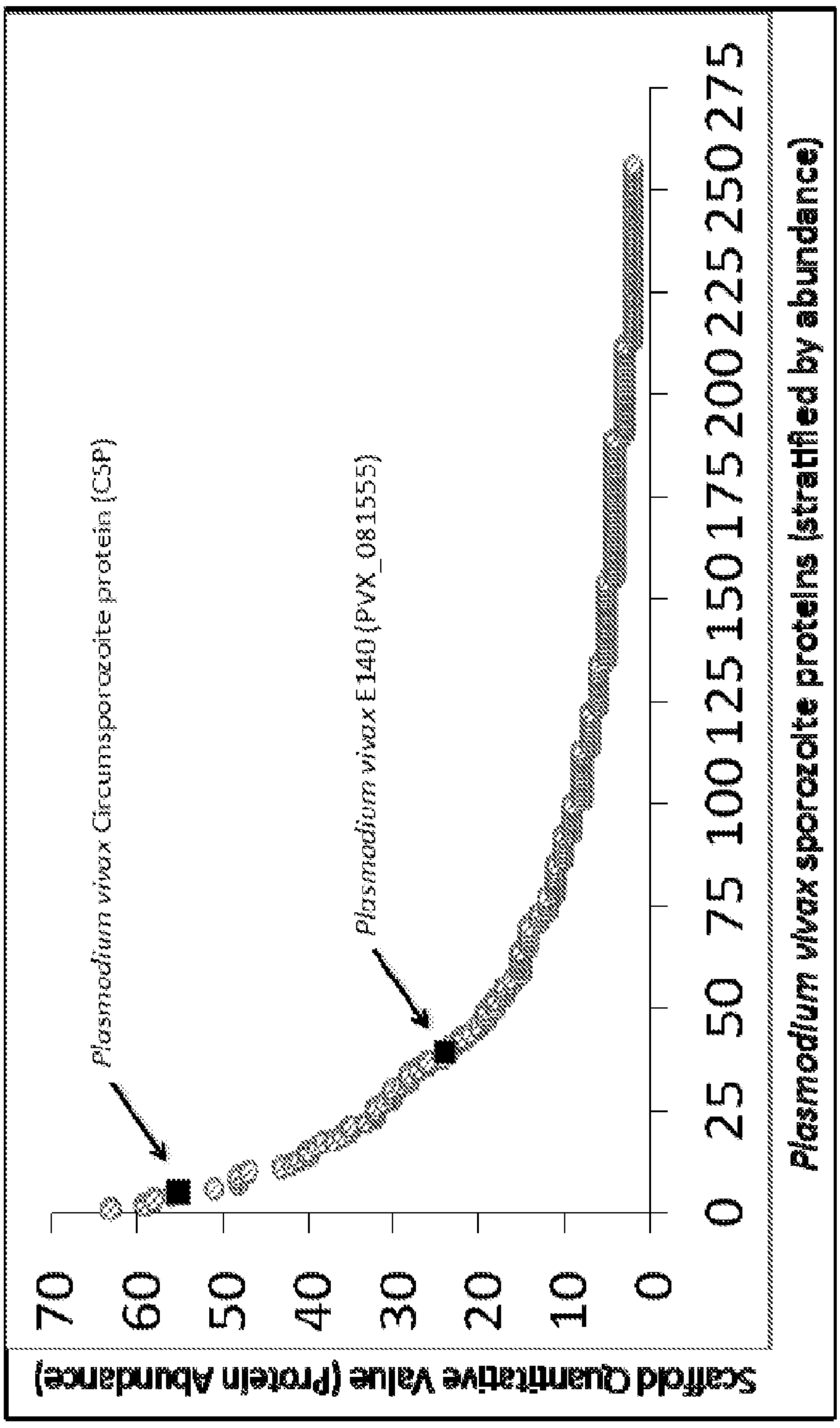
Plasmodium vivax Circumsporozoite protein (CSP)
Plasmodium vivax E140 (PVX_081555)
0 25 50 75 100 125 150 175 200 225 250 275
Plasmodium vivax sporozoite proteins (stratified by abundance)
0 10 20 30 40 50 60 70
Scaffold Quantitative Value (Protein Abundance)

FIG. 15