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(54) Title: THERAPEUTIC CANCER VACCINE TARGETED TO HAAH (ASPARTYL-[ASPARAGINYL]-BETA-HYDROXYLASE)

(57) Abstract: The present invention encompasses a cancer vaccine therapy targeting Aspartyl-[Asparaginyl]-β- hydroxylase (HAAH). The present invention contemplate bacteriophage expressing HAAH peptide fragments and methods for using said bacteriophage in methods of treating cancer.

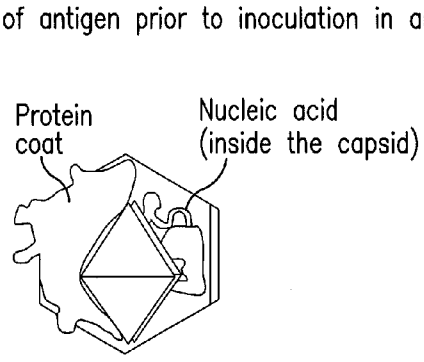


FIG. 1

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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Therapeutic Cancer Vaccine Targeted to HAAH (Aspartyl-[Asparaginyl]-beta-Hydroxylase)

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Serial No. 13/386,487 filed March 15, 2013, the disclosure of which is hereby incorporated in its entirety.

BACKGROUND OF THE INVENTION

Cancer is one of the most devastating diseases both in terms of human life opportunity loss and health care cost. It also presents unmet clinical needs. Currently available chemotherapies have limited efficacy and limited target patient population. Even the successful immunotherapies have shortcomings similar to chemotherapies. Moreover, essentially all cancer therapeutics have significant adverse side effects.

Aspartyl-(Asparaginyl)-β-hydroxylase (HAAH) is over expressed in various malignant neoplasms, including hepatocellular and lung carcinomas. HAAH is a tumor specific antigen, which is specifically expressed on the surface of certain malignant cells. HAAH is a hydroxylation enzyme that modifies factors such as Notch that contribute to cancer etiology by causing cell proliferation, motility, and invasiveness. Neutralizing the enzyme or reducing its expression leads to normal phenotype(s) in cancer cells. Anti-HAAH antibodies (as well as siRNA) have been shown to be cytostatic. An all-human sequence anti-HAAH (PAN- 622) has shown to inhibit tumor growth by more than 90% in animal studies by passive immunotherapy. However, HAAH is well conserved and is also over expressed in placenta hence it is not sufficiently immunogenic in animals and it is certainly a self antigen in humans.

A vaccine therapy targeted to a pan-cancer-specific antigen such as HAAH that has proven relevance to cancer etiology is very desirable. Its economic impact will be enormous both in terms of job creation and increased productivity as well as in savings in health care and extending productive lives. The vaccine therapy of the present invention is novel both in terms of its target and the vaccine entity.

SUMMARY OF THE INVENTION

The present invention encompasses a cancer vaccine therapy targeting human Aspartyl-[Asparaginyl]-β-hydroxylase (HAAH).

Certain embodiments of the present invention contemplate bacteriophage expressing HAAH peptide fragments, wherein the bacteriophage may be any one of Lambda, T4, T7, or M13/f1.

The present invention further contemplates methods of treating cancer comprising stimulating the immune system of a patient with bacteriophage expressing HAAH fragments.

The present invention also contemplates nano-particles comprising at least one amino acid sequence native to HAAH.

The present invention also encompasses methods for treating cancer comprising the step of providing an immune system stimulating amount of a Lambda phage to a patient, wherein the Lambda phage comprises amino acid sequences native to HAAH expressed on its surface.

The present invention also encompasses methods for treating cancer comprising the step of providing an immune system-stimulating amount of a nano-particle to a patient, wherein the nano-particle comprises amino acid sequences native to HAAH.

One embodiment of the present invention contemplates bacteriophage comprising at least one amino acid sequence native to HAAH, wherein the at least one amino acid sequence native to HAAH is selected from the group consisting of the amino acid sequence of Construct I, the amino acid sequence of Construct II and the amino acid sequence of Construct III.

The present invention also contemplates a Lambda phage expressing the amino acid sequence of Construct I, the amino acid sequence of Construct II or the amino acid sequence of Construct III on its surface.

Embodiments of the present invention also contemplate nucleic acid construct comprising at least one nucleotide sequence encoding an amino acid sequence native to HAAH and a nucleic acid sequence encoding bacteriophage lambda head decoration protein D (hereinafter "gpD").

Another embodiment of the present invention includes nucleic acid constructs comprising nucleotide sequences encoding the amino acid sequence of Construct I, the amino acid sequence of Construct II or the amino acid sequence of Construct III.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a representation of the *in vitro* expression of an antigen.

Figure 2 provides a comparison of AAH amino acid sequences.

Figure 3 is a graph that demonstrates the efficacy of an antibody against HAAH in liver cancer cells.

Figure 4 shows the mechanism of immunization in accordance with the present invention.

Figure 5 shows the immune response.

Figure 6 A-B Homologous recombination of donor plasmid pAN-A- with recipient phage vector. Only some of the lambda genes are shown. The unique Nhe I and Bssh II site in the lambda genome used for cloning is shown as is lacZa, a DNA cassette comprised of lacPO, RBS and the first 58 codons of lacZ. Generated recombinant phages are designated as HAAH construct I, II and III which contains an insert of HAAH fragment. Only diagram of construct I is shown here. The insert is fused with gpD head protein gene of lambda to produce gpD-HAAH construct I fusion on lambda capsid. The maps are not to scale.

Figure 7 diagrams one method of growing and purifying constructs in accordance with the present invention.

Figure 8 is an example of a Western blot HAAH- vaccine screening for a cancer vaccine candidate.

Figure 9 shows FB50 MAb blotted to HAAH constructs in accordance with the present invention.

Figure 10 shows ELISA results.

Figure 11 This scatter chart shows the result of HAAH test as a cancer biomarker on a group of 857 individuals composed of 211 individuals known not to have cancer and 646 patients who are diagnosed with cancer. The cancer group is composed of a mix of individuals with different types of cancer (Breast, Prostate, Lung, Colon) in various stages from one to four. Combining the 12 false positive and 34 false negative results, the test has less than 5.4% error even in such a large group of patients. Horizontal axis is the patient index.

Figure 12 shows tumor challenge data regarding tumor volume.

Figure 13 shows tumor challenge data regarding tumor volume.

Figure 14 shows tumor challenge data regarding tumor volume.

Figure 15 provides mouse sera data in relation to one embodiment of the present invention.

Figure 16 provides mouse sera data in relation to one embodiment of the present invention.

Figure 17 provides data regarding immunogenicity relative to dose.

Figure 18 shows a Western Blot of FB50 and HAAH Sera.

Figure 19 shows amino acid sequences in accordance with an embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

For simplicity and illustrative purposes, the principles of the present invention are described by referring to various exemplary embodiments thereof. Although the preferred embodiments of the invention are particularly disclosed herein, one of ordinary skill in the art will readily recognize that the same principles are equally applicable to, and can be implemented in other systems, and that any such variation would be within such modifications that do not part from the scope of the present invention. Before explaining the disclosed embodiments of the present invention in detail, it is to be understood that the invention is not limited in its application to the details of any particular arrangement shown, since the invention is capable of other

embodiments. The terminology used herein is for the purpose of description and not of limitation. Further, although certain methods are described with reference to certain steps that are presented herein in certain order, in many instances, these steps may be performed in any order as would be appreciated by one skilled in the art, and the methods are not limited to the particular arrangement of steps disclosed herein.

The present invention is based on the discovery that bacteriophage surface-expressed HAAH is highly immunogenic and could overcome tolerance of self antigen because of altered presentation and the adjuvant function of bacteriophage itself. The present invention provides a cancer vaccine therapy targeting HAAH using bacteriophage-expressed HAAH fragments.

It has been shown that passive immunotherapy using an all-human anti-HAAH is effective in cellular and animals models of cancer (in nude mice model, Figure 1). The present invention demonstrates that bacteriophage delivery of HAAH fragments as vaccine can overcome the problem of self antigen tolerance by providing novel antigen presentation and inherent phage adjuvant properties.

In vitro activation of dendritic cells by tumor antigens, prior to administration to patient body shows promising results for cancer therapy. Unfortunately the process is cumbersome, expensive and time consuming for mass scale immune therapy against various cancers. Bacteriophage display is a simple way of achieving favorable presentation of peptides to the immune system. Previous findings revealed that recombinant bacteriophage can prime strong CD8+ T lymphocytes (CTLs) responses both in vitro and in vivo against epitopes displayed in multiple copies on their surface, activate T-helper cells and elicit the production of specific antibodies all normally without adjuvant.

As proposed herein, vaccination with lambda phage-displaying cancer specific antigen such as HAAH has a number of potential advantages. One of the advantages is display of multiple copies of peptides on the same lambda phage, and once the initial phage display has been made, subsequent production should be far easier and cheaper than the ongoing process of coupling peptides to carriers. There is also good evidence that due to particulate nature, phage-displayed peptides can access both the major histocompatibility complex (MHC) I and MHC II pathway,

suggesting lambda phage display vaccines can stimulate both cellular and humoral arms of the immune system, although as extra cellular antigens, it is to be expected that the majority of the responses will be antibody (MHC class II) biased. It has been shown that particulate antigens, and phage in particular, can access the MHC I pathway through cross priming, and it is likely that it is this process which is responsible for stimulating a cellular response. This added cellular response mediated by CD8+ T cells helps to eliminate the cancer cells. Also, the role of Innate immunity in cancer is well established fact. Lambda phage can also act as nonspecific immune stimulators. It is likely that a combination of the foreign DNA (possibly due to the presence of CpG motifs) and the repeating peptide motif of the phage coat are responsible for the nonspecific immune stimulation. As a summary: whole lambda phage particles possess numerous intrinsic characteristics which make them ideal as vaccine delivery vehicles. For use as phage display vaccines, the particulate nature of phage means they should be far easier and cheaper to purify than soluble recombinant proteins since a simple centrifugation/ultra-filtration and column chromatography step should be sufficient to remove the majority of soluble contaminants. Additionally, the peptide antigen comes already covalently conjugated to an insoluble immunogenic carrier with natural adjuvant properties, without the need for complex chemical conjugation and downstream purification processes which must be repeated with each vaccine batch.

The present invention provides a prophylactic and therapeutic "phage vaccine" for both cancer prevention and treatment. In the present invention, fragmented HAAH peptides are successfully displayed on the surface of lambda head and large scale production and purification is carried out to perform animal experiments. The detail of these procedures is depicted below.

A. Construction of bacteriophage lambda for display of HAAH peptides:

We designed a bacteriophage lambda system to display HAAH peptides fused at the C terminus of the head protein gpD of phage lambda. Molecular analysis of HAAH reveals a partial amino terminal homology of this protein with other two proteins called Junctin and Humbug. The role of these other two proteins in human physiology is not known completely. To avoid any complication such as activating immune system against these homologous proteins, we specifically eliminated these sequences from our phage display constructs. For proper display of HAAH peptides on lambda head, the rest of the HAAH sequence is segmented in three sections.

They are designated as HAAH construct 1, HAAH construct 2 and HAAH construct 3 (see Figure 7). Using HAAH specific oligo primers these segments are amplified from the HAAH gene which was previously cloned in our laboratory for expression in baculovirus system. The oligo sequence of each PCR primer is modified slightly to produce Nhe I and Bssh II restriction sites in each end of amplified HAAH segments. After restriction digestion, these segments are inserted separately at the NheI-BsshII site of the 3' end of a DNA segment encoding gpD under the control of the lac promoter. The constructs are created in a plasmid vector (donor plasmid pAN-A), which also carries loxPwt and loxP511 sequences. Cre-expressing cells (E. coli) are transformed with these recombinant plasmids and subsequently infected with a recipient lambda phage that carries a stuffer DNA segment flanked by loxPwt and loxP511 sites. Recombination occurs in vivo at the lox sites and Ampr cointegrates are formed (Figure 2), which are spontaneously lyse the E. coli and released in culture media. The cointegrates produce recombinant phages that display HAAH peptides fused at the C terminus of gpD. Approximately 200 copies of these peptides are displayed on a single phage head.

B. Selection of lambda cointegrates and production of recombinant phages which display HAAH peptides:

Lambda cointegrates are selected on Luria Bartani (LB) ampicillin agar (100ug/ml amp, 15% agar) plates. Briefly, spontaneously lysed E. coli culture is used to infect Cre-ve E. coli cells and spread on LB ampicillin agar plates. Plates are incubated at 32 ° C for 48 hours to obtain Ampr colonies. These Ampr colonies are immune to super infection and carry the phages as plasmid cointegrates. The Ampr colonies containing the lambda cointegrate are grown separately at LB Ampicillin (100ug/ml) at 37 ° C for four hours. Lambda phages are spontaneously induced in these cultures and result in complete lysis. This cell free supernatant is used to infect E. coli cells and plated on solid LB agar (15%) plate to obtain phage plaques. The resulting phage plaques are harvested from the plate and single plaques are purified three times on E. coli by the standard procedures described by Sambrook et al.

C. Conformation of lambda cointegrates containing HAAH fragments:

All bacterial colonies, containing lambda cointegrates, which are used for HAAH phage vaccine production are verified by PCR. In this process the presence of each cloned inserts in bacterial

colonies are confirmed by PCR amplification of HAAH specific insert DNA by XbaI-5/(TTGGTTAGCAAGTTAATACC) and XbaI-3/ (TAGATTTGAATGACTTCCCC) primer set. These two specific primers flank the unique Xba I site of lambda genome and used for PCR the complete insert presence in between Lox recombination sites of lambda DNA.

D. Growth and purification of recombinant phages displaying HAAH peptides:

Growth of the plaque purified phages is performed in two steps. The steps are designated as plate lysate method and large scale liquid lysate method. The detail of these procedures are described in Sambrook et al. The lysed culture is chilled at room temperature for further purification by liquid column chromatography technique.

E. Large scale purification of recombinant Lambda-constructs using Column chromatography technique:

CIM® monolithic columns are an ideal chromatographic support for purifying large biomolecules and nanoparticles, bacterial viruses and plasmid DNA. The pore size of these monolithic columns are adjusted to accommodate even the largest molecules and optimized for very high binding capacities at the highest flow rates. We adopted these monolithic columns for large scale purification of lambda phages displaying HAAH-peptides. In order to obtain infective virus during purification process we investigated chemical conditions that provided the maximal yield of phage and which also preserved high infectivity. This information is necessary to adjust chromatographic methods accordingly to avoid undesired phage deactivation during processing.

HPLC equipment: All experiment is preformed on a gradient AKTA purifier FPLC chromatography system (GE Healthcare) equipped with Unicorn 5.1 chromatography software, P-900 FPLC pumps, UPC-900 variable wavelength detector, and FRAC-920 fraction collector. CIM ion exchange chromatography is monitored for UV at 280 nm as well as for conductivity and the gradient profile, associated with marks for point of injection and fraction number. Stationary phase: A strong anion exchange (quaternary amine-QA) methacrylate- based CIM

disk monolithic column (BIA Separations, Ljubljana, Slovenia) is used for this purification procedure. Mobile phase: 125 mM NaH_2PO_4, pH 7.0 (loading buffer) and 125 mM NaH_2PO_4, 1.5 M NaCl, pH 7.0 (elution buffer) of different pH values is used. All buffers is filtered through 0.22 micron pore size filters before use. These strong anion exchange (quaternary amine-QA) methacrylate- based CIM disk monolithic columns is periodically sanitized after processing, by a 2 hour procedure using 1 M NaOH. Processing of phage lysate for QA column analysis: Phage lysates (10 mL) are centrifuged at 12000 x g for 10 minutes at 4 ° C and the phage containing supernatant is filtered through a 0.22 micron filter prior to loading the phage on the column for chromatography. Collected fractions of 1 mL are analyzed via plaque assay to determine presence of infective phage. Plaque assay data is analyzed to optimize specific conditions for column chromatography purification of display phages. When larger amounts of highly concentrated phage will be required, the linear gradient will be changed into a stepwise gradient where narrower peaks will be achieved and fraction collection will be easier. Based on data from the linear gradient, we will introduce conditions for the stepwise gradient for large scale purification of display phages.

F. Immunoblot and Western blot analysis of recombinant Lambda-constructs:

To verify the expression of fusion-peptides on lambda head, immunoblot and Western blot analysis are carried out.

For immunoblot assay each phage constructs are separately plated on LB agar plate to obtain 100 to 150 plaques in each plate. The plates are incubated at 37 ° C for 18 hours, until the plaques are about one mm in size. Next, a 137 mm colony/plaque screen membrane (NEN® Research products, Boston, MA) is soaked in distilled water and blotted dry on a filter paper. This membrane is carefully placed on the top agar and incubation was continued at 37 ° C for another 15 minutes. The membrane is peeled from the agar, and washed three times with Tris saline to remove the debris and bacteria. The plates are then stored at 4 ° C and the washed NEN membranes are blocked with 2% casein solution for 1 hour. After blocking, the membranes are incubated in a casein solution containing 1.25 ug/ml of diluted FB 50 monoclonal antibody. This FB50 HAAH specific monoclonal antibody was previously generated in our laboratory for diagnostic application of prostate cancer. After incubation at room temperature for two hours the membranes are washed twice in Tris saline with 0.05% Triton X-100, and once in Tris saline for

15 minutes each. The monoclonal treated membranes are incubated with 2.0 μg/ml of alkaline phosphatase labeled rabbit antimouse IgG (Kirkegaard and Perry) for one hour at room temperature. The membranes are consecutively washed three times in the same way described earlier in this procedure, followed by a final wash with 0.9% NaCl. Finally the membranes are treated with Fast Red and naphthol substrate solution for about 10 minutes and the reaction was stopped by washing the membrane in distilled water. The pink immunoreactive spots corresponds the recombinants expressing HAAH specific peptides on lambda head. For Western blots, purified lambda phage particles were electrophoresed under reducing conditions on 0.1% (w/v) SDS/10% polyacrylamide gel followed by electrobloting onto PVDF membrane (Immobilon, Millipore, Bedford, MA). Fusion proteins are detected either 2.5 ug/ml diluted rabbit polyclonal sera raised against recombinant expressed lambda GpD or HAAH specific E6 mouse monoclonal antibody (final concentration 1.25 ug/ml). The rabbit antisera treated membranes are incubated with 2.0 μg/ml of alkaline phosphatase labeled goat anti-rabbit IgG and mouse monoclonal treated membranes are incubated with 2.0 μg/ml of alkaline phosphatase labeled rabbit antimouse IgG for one hour at room temperature. The membranes are consecutively washed three times in the same way described earlier in plaque lift assay. Finally the membranes are treated with Fast Red and naphthol substrate solution for about 10 minutes and the reaction is stopped by washing the membrane in distilled water. immunoreactive lines correspond to the gpD specific recombinant proteins.

Animal Experiments to Evaluate Antigenic Nature of HAAH Phage Vaccine:

A. Study of antigenicity of HAAH-phage vaccine on Female BALB/c mice mice.

The purpose of this experiment is to determine the efficacy of HAAH-phage vaccine to elicit antibody response in BALB/c female mice. Previously three separate HAAH-lambda phage constructs were prepared where fragmented HAAH antigens are displayed on surface of lambda phage head as fusion of lambda capsid protein gpD. Such three constructs were designated as HAAH construct 1, HAAH construct 2, and HAAH construct 3. Four separate groups of mice (Group A, Group B, Group C, 5 mice in each group and Group D, 40 mice) will be injected subcutaneously (s/c) with various HAAH phage constructs as described in chart below (Chart 1). Briefly, group A mice will receive 5×10^8 pfu of HAAH construct 1 phage particles suspended in 500 μl of sterile PBS. Similarly group B and group C mice will receive same quantity of

HAAH construct 2 and HAAH construct 3 phage particles respectively. Group D mice will receive equi-molar mixture of all 3 phage constructs. A fifth group of mice (group E, 40 mice) will receive recombinant HAAH antigen (50 μg/mice) suspended in sterile PBS. As a control (group F, 40 mice) will be injected with wild type phage pAN-A-λ. After primary inoculation, mice will receive 1st and 2nd booster (dose will be the same as primary inoculation) of corresponding antigens at 2 weeks interval. All animals will be bled prior primary inoculation. Serum samples will be collected before every booster to monitor progression of immune response against HAAH antigens. After 21 days animal will be euthanized for final bleeding through cardiac puncture. Finally animals will be sacrificed by spinal dislocations. Sera from group D, group E and group F animals will be saved at -70oC freezer for second animal experiment. During experiment, all animals will be monitored for their health conditions. The immune response against various HAAH-phage vaccines will be monitored by western immunoblot and ELISA.

	Groups						
Days	A	B	C	D	E	F	Scoring
0	HAAH construct 1 5×10^8 pfu	HAAH construct 2 5×10^8 pfu	HAAH construct 3 5×10^8 pfu	Mixture of 3 HAAH constructs 5×10^8 pfu	Recombinant HAAH 50 μg	pAN-A-λ 5×10^8 pfu	*
7	HAAH construct 1 5×10^8 pfu	HAAH construct 2 5×10^8 pfu	HAAH construct 3 5×10^8 pfu	Mixture of 3 HAAH constructs 5×10^8 pfu	Recombinant HAAH 50 μg	pAN-A-λ 5×10^8 pfu	
14	HAAH construct 1 5×10^8 pfu	HAAH construct 2 5×10^8 pfu	HAAH construct 3 5×10^8 pfu	Mixture of 3 HAAH constructs 5×10^8 pfu	Recombinant HAAH 50 μg	pAN-A-λ 5×10^8 pfu	
21	Final Bleed	Final Bleed	Final Bleed	Final Bleed	Final Bleed	Final Bleed	* For 21 days

Scoring: 0-normal, 1-lethargy and ruffled fur, 2-lethargy, ruffled fur and hunchback, 3-lethargy, ruffled fur, hunchback, and partially closed eyes, 4-moribund, 5-dead.

B. Evaluation of Humoral Immunity Response Against HAAH Phage Constructs:

Previously in xenograft models of human primary liver cancer, the initial target disease, treatment with anti-HAAH antibodies reduced cancer tumor size in all animals, and in 75% of cases after four weeks of treatment tumors were kept to a non-detectable size. In a model of tumor metastasis using human colon cancer cells spreading to the liver, treatment with anti-

HAAH antibodies greatly reduced the number and size of metastases. These results are highly significant and clearly indicate the utility of anti-HAAH in the treatment of human cancer. It is noteworthy that in both these instances animals were treated with antibody alone, not in conjunction with any other treatment. In this experiment, 4 groups of nude mice (Group A, Group B and Group C, and group D, 5 mice in each group) will be injected subcutaneously with a primary human liver cancer in their left flank. After 72 hours Group A, Group B and Group C nude mice will be treated by intraperitonial (i/p) route with 300 ul of sera previously collected from Group D, Group E and Group F mice of 1st animal experiment respectively. As a control Group D nude mice will be receive 300 ul of PBS. The treatment will continue every 48 hours for an additional 4 weeks. After that, the animal will be monitored for another 2 weeks without any intervention. The progression of the tumor will be monitored in treated and control groups every 48 hours to evaluate the result. Finally animals will be sacrificed by spinal dislocations and their organ will be examined by a pathologist for metastasis.

Example 1

Presently, we have designed, developed and produced and lambda-phage based therapeutic anticancer vaccine (nanoparticle) targeting human (Asparaginyl)-β-hydroxylase (HAAH). To overcome the self-antigen tolerance of the molecule, we have designed a vaccine entity that contains an immunostimulant and present the HAAH in a manner that is unfamiliar to the body. We have expressed three portions of the HAAH protein, sequences from the N-terminus, middle portion, and C-terminus as fusion proteins (with the gpD bacteriophage antigen) on the surface of bacteriophage lambda, generating 200-300 copies per phage. These vaccine entities were characterized and are readily and routinely produced at a level of 1012 plaque forming units (pfu) per liter of E. coli culture. The bacteriophage vaccines have been successfully isolated and purified using tangential flow filtration, a highly scalable process, as well as by PEG precipitation followed by exhaustive dialysis. Both of these processes have reduced bacterial endotoxins to levels within FDA guidelines for formulated human doses. The ease and yield of the manufacturing process allow production of approximately 100 human doses (based on anticipated dose requirements for immunogenicity in human subjects) per liter of culture. The bacteriophage can be rendered non-infectious by ultraviolet radiation; hence it is referred to as nano-particle based vaccine.

Table 1: Growth and Purification Data for HAAH Lambda Phage Constructs

HAAH Constructs	HAAH-1A	HAAH-2A	HAAH-3A
Amount Phage in Culture (pfu/L)	3.5×10^{12}	2.7×10^{12}	5.7×10^{12}
Purified Phage (pfu/L) and Yield	2.1×10^{12} (58%)	1.9×10^{12} (71%)	4.0×10^{12} (70%)
UV-treated Phage (pfu/L)	0	----	0
Nanoparticles/L (infectious +non-infectious phage)	4.6×10^{13}	9.1×10^{13}	8.6×10^{12}
Endotoxin (EU/10^8 phage)	156	140	4

Example 2

All three entities display highly significant, dose dependent immunogenicity as assessed by antibody ELISAs. To evaluation the therapeutic effect of the nanoparticle vaccine, we initiated tumor formation in BALB/c mice using a mouse hepatocellular carcinoma line BNLT3. This cell line, a highly tumorigenic subclone of the ATCC cell line, BNL 1ME A.7R.1, was developed by J. Wands at the Liver Research Center, Rhode Island Hospital by 3 serial subcutaneous passages of the parental cell cline. On Day 0 of the tumor challenge study, 4 groups of 5 mice each were administered 5 E03 BNLT3 cells subcutaneously. On the same day, the animal received the first of three weekly subcutaneous doses (at 0, 7, and 14 days) of nanoparticle vaccine as monovalent vaccines of each of the three bacteriophage constructs (10E10 pfu/dose) or a buffer control. The mice were then observed for tumor growth and tumor volume was determined. After 4 weeks (Study Day 28), 3 of 5 mice in the control group had measurable tumor growth, while in the HAAH N-terminus, middle portion, and C-terminus construct vaccine groups, 0/5, 2/5, and 0/5 animals, respectively, had measureable tumor growth. The mean tumor volumes of the 5 animals in each of the two groups with tumor growth were 85.8 mm^3 for the control group and 24.9 mm^3 (29% of the control tumor volume) for the HAAH middle portion group. Overall, the vaccinated groups had 2/15 animals and mean tumor growth of 8.3 mm^3 (>10% of the control group tumor size).

Example 3

Immunocompetent mice were used to test immunogenicity of three phage-based vaccine candidates, encompassing the N-terminal, mid, and C-terminal protions of the HAAH extracellular domain. All three entities display highly significant, dose-dependent immunogenicity. Animals were injected with 5x107-5x109 pfus on days 0, 7, and 14. Animals were bled on day 21 and immunogenicity was screened using recombinant HAAH in an ELISA format. Cell-based ELISAs using liver (FOCUS) and lung (H460) cancer cell lines as well as FACS analysis on these lines were performed. The immunized mice sera had clear anti-HAAH (or anti-cance cell) activity in all tests. Immunogenicity was dose and construct dependent. This work demonstrates that a nano-particle, phage-based vaccine can break immune tolerance to the native HAAH protein and elicit a specific antibody response; incicating that such vaccines may have significant therapeutic value.

While the invention has been described with reference to certain exemplary embodiments thereof, those skilled in the art may make various modifications to the described embodiments of the invention without departing from the scope of the invention. The terms and descriptions used herein are set forth by way of illustration only and not meant as limitations. In particular, although the present invention has been described by way of examples, a variety of compositions and processes would practice the inventive concepts described herein. Although the invention has been described and disclosed in various terms and certain embodiments, the scope of the invention is not intended to be, nor should it be deemed to be, limited thereby and such other modifications or embodiments as may be suggested by the teachings herein are particularly reserved, especially as they fall within the breadth and scope of the claims here appended. Those skilled in the art will recognize that these and other variations are possible within the scope of the invention as defined in the following claims and their equivalents.

What is claimed is:

1. A bacteriophage comprising at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase.

2. The bacteriophage of claim 1, wherein the at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase is selected from the group consisting of the amino acid sequence of Construct I.

3. The bacteriophage of claim 1, wherein the bacteriophage comprises the amino acid sequence of Construct II.

4. The bacteriophage of claim 1, wherein the bacteriophage comprises the amino acid sequence of Construct III.

5. The bacteriophage of claim 1, wherein the bacteriophage is selected from the group consisting of Lambda, T4, T7, and M13/f1.

6. The bacteriophage of claim 5, wherein the bacteriophage is bacteriophage Lambda.

7. A method for treating cancer comprising the step of providing a patient with an immune system stimulating amount of the bacteriophage of claim 1.

8. A nucleic acid construct comprising at least one nucleotide sequence encoding an amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase and a nucleotide sequence encoding gpD.

9. The nucleic acid construct of claim 8, wherein the at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase is the amino acid sequence of Construct I.

10. The nucleic acid construct of claim 8, wherein the at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase is the amino acid sequence of Construct II.

11. The nucleic acid construct of claim 8, wherein the at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase is the amino acid sequence of Construct III.

12. A recombinant Lambda phage comprising the nucleic acid construct of claim 8.

13. A composition comprising nano-particles, wherein the nano-particles further comprise at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase.

14. The composition of claim 13, wherein the at least one amino acid sequence native to Aspartyl-[Asparaginyl]-β-hydroxylase is the amino acid sequence of Construct I.

15. The composition of claim 13, wherein the nano-particle comprises the amino acid sequence of Construct II.

16. The composition of claim 13, wherein the nano-particle comprises the amino acid sequence of Construct III.

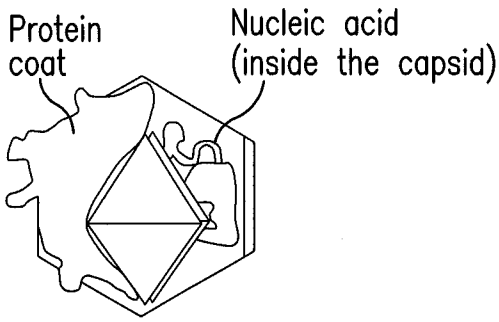
17. A method for treating cancer comprising the step of providing a patient with an immune system stimulating amount of the composition of claim 13.

18. A method for treating cancer comprising the step of contacting dendritic cells of a patient with an immune system stimulating amount of the composition of claim 13.

19. A method for treating cancer comprising the step of providing an immune system stimulating amount of Lambda phage to a patient, wherein the Lambda phage comprise amino acid sequences native to Aspartyl-[Asparaginyl]-β-hydroxylase expressed on their surface.

20. The method of claim 19, wherein the amino acid sequences native to Aspartyl-[Asparaginyl]-β-hydroxylase comprise the amino acid sequence of Construct I, the amino acid sequence of Construct II and the amino acid sequence of Construct III.

In vitro expression of antigen prior to inoculation in animals



Designing lambda system to display antigenic proteins as a C terminus fusion product of gpD

FIG. 1

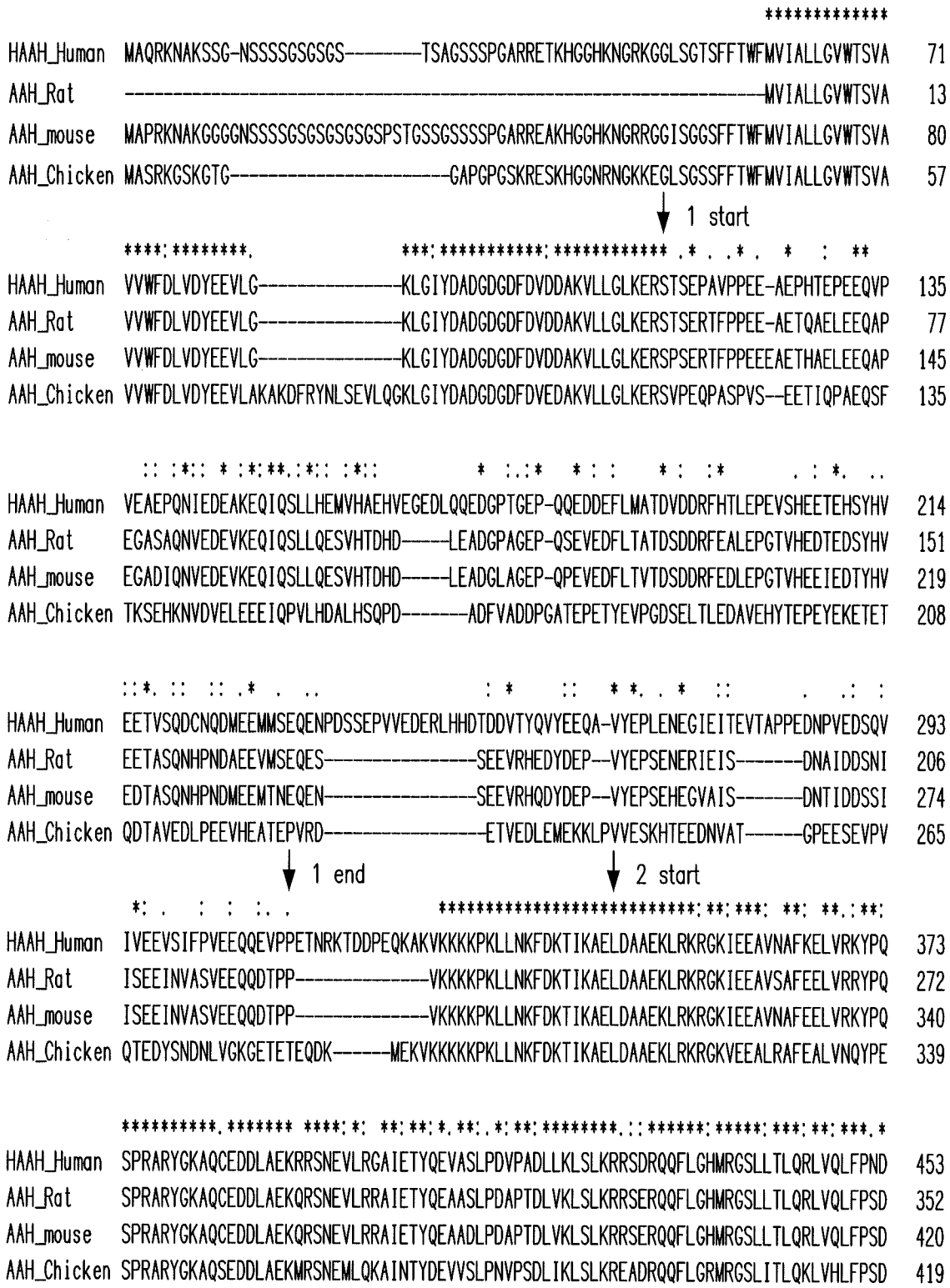
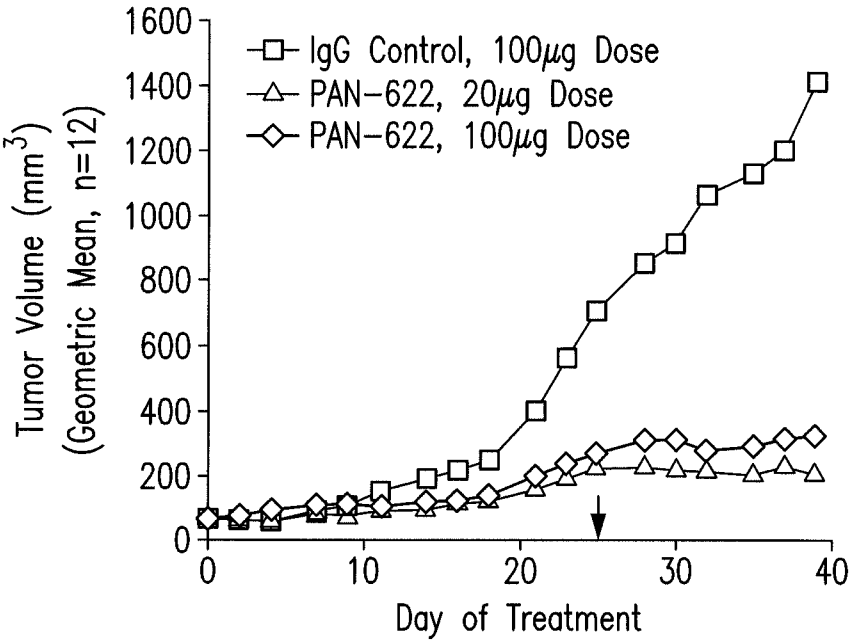

HAAH_Human MAQRKNAKSSG-NSSSSGSGSGS--------TSAGSSSPGARRETKHGGHKNGRKGGLSGTSFFTWFMVIALLGVWTSVA 71
AAH_Rat ----------MVIALLGVWTSVA 13
AAH_mouse MAPRKNAKGGGGNSSSSGSGSGSGSGSPSTGSSGSSSSPGARREAKHGGHKNGRRGGISGGSFFTWFMVIALLGVWTSVA 80
AAH_Chicken MASRKGSKGTG----------GAPGPGSKRESKHGGNRNGKKEGLSGSSFFTWFMVIALLGVWTSVA 57
1 start
HAAH_Human VVWFDLVDYEEVLG----------KLGIYDADGDGDFDVDDAKVLLGLKERSTSEPAVPPEE-AEPHTEPEEQVP 135
AAH_Rat VVWFDLVDYEEVLG----------KLGIYDADGDGDFDVDDAKVLLGLKERSTSERTFPPEE-AETQAELEEQAP 77
AAH_mouse VVWFDLVDYEEVLG----------KLGIYDADGDGDFDVDDAKVLLGLKERSPSERTFPPEEEAETHAELEEQAP 145
AAH_Chicken VVWFDLVDYEEVLAKAKDFRYNLSEVLQGKLGIYDADGDGDFDVEDAKVLLGLKERSVPEQPASPVS--EETIQPAEQSF 135
HAAH_Human VEAEPQNIEDEAKEQIQSLLHEMVHAEHVEGEDLQQEDGPTGEP-QQEDDEFLMATDVDDRFHTLEPEVSHEETEHSYHV 214
AAH_Rat EGASAQNVEDEVKEQIQSLLQESVHTDHD-----LEADGPAGEP-QSEVEDFLTATDSDDRFEALEPGTVHEDTEDSYHV 151
AAH_mouse EGADIQNVEDEVKEQIQSLLQESVHTDHD-----LEADGLAGEP-QPEVEDFLTVTDSDDRFEDLEPGTVHEEIEDTYHV 219
AAH_Chicken TKSEHKNVDVELEEEIQPVLHDALHSQPD-------ADFVADDPGATEPETYEVPGDSELTLEDAVEHYTEPEYEKETET 208
HAAH_Human EETVSQDCNQDMEEMMSEQENPDSSEPVVEDERLHHDTDDVTYQVYEEQA-VYEPLENEGIEITEVTAPPEDNPVEDSQV 293
AAH_Rat EETASQNHPNDAEEVMSEQES----------SEEVRHEDYDEP--VYEPSENERIEIS-------DNAIDDSNI 206
AAH_mouse EDTASQNHPNDMEEMTNEQEN----------SEEVRHQDYDEP--VYEPSEHEGVAIS-------DNTIDDSSI 274
AAH_Chicken QDTAVEDLPEEVHEATEPVRD----------ETVEDLEMEKKLPVVESKHTEEDNVAT------GPEESEVPV 265
1 end
2 start
HAAH_Human IVEEVSIFPVEEQQEVPPETNRKTDDPEQKAKVKKKKPKLLNKFDKTIKAELDAAEKLRKRGKIEEAVNAFKELVRKYPQ 373
AAH_Rat ISEEINVASVEEQQDTPP----------VKKKKPKLLNKFDKTIKAELDAAEKLRKRGKIEEAVSAFEELVRRYPQ 272
AAH_mouse ISEEINVASVEEQQDTPP----------VKKKKPKLLNKFDKTIKAELDAAEKLRKRGKIEEAVNAFEELVRKYPQ 340
AAH_Chicken QTEDYSNDNLVGKGETETEQDK------MEKVKKKKKPKLLNKFDKTIKAELDAAEKLRKRGKVEEALRAFEALVNQYPE 339
HAAH_Human SPRARYGKAQCEDDLAEKRRSNEVLRGAIETYQEVASLPDVPADLLKLSLKRRSDRQQFLGHMRGSLLTLQRLVQLFPND 453
AAH_Rat SPRARYGKAQCEDDLAEKQRSNEVLRRAIETYQEAASLPDAPTDLVKLSLKRRSERQQFLGHMRGSLLTLQRLVQLFPSD 352
AAH_mouse SPRARYGKAQCEDDLAEKQRSNEVLRRAIETYQEAADLPDAPTDLVKLSLKRRSERQQFLGHMRGSLLTLQRLVQLFPSD 420
AAH_Chicken SPRARYGKAQSEDDLAEKMRSNEMLQKAINTYDEVVSLPNVPSDLIKLSLKREADRQQFLGRMRGSLITLQKLVHLFPSD 419

FIG. 2



FIG. 2 (Continued)



- Tumors established Day-3 with 10^7FOCUS cells (hepatic cancer cell line)
- Antibody injected IV 3X/wk, Days 0-25 (last injection marked with arrow)
- 5 of 12 PAN-622 treated animals reached undetectable tumor volume, a direct evidence of cytotoxic effect

FIG. 3

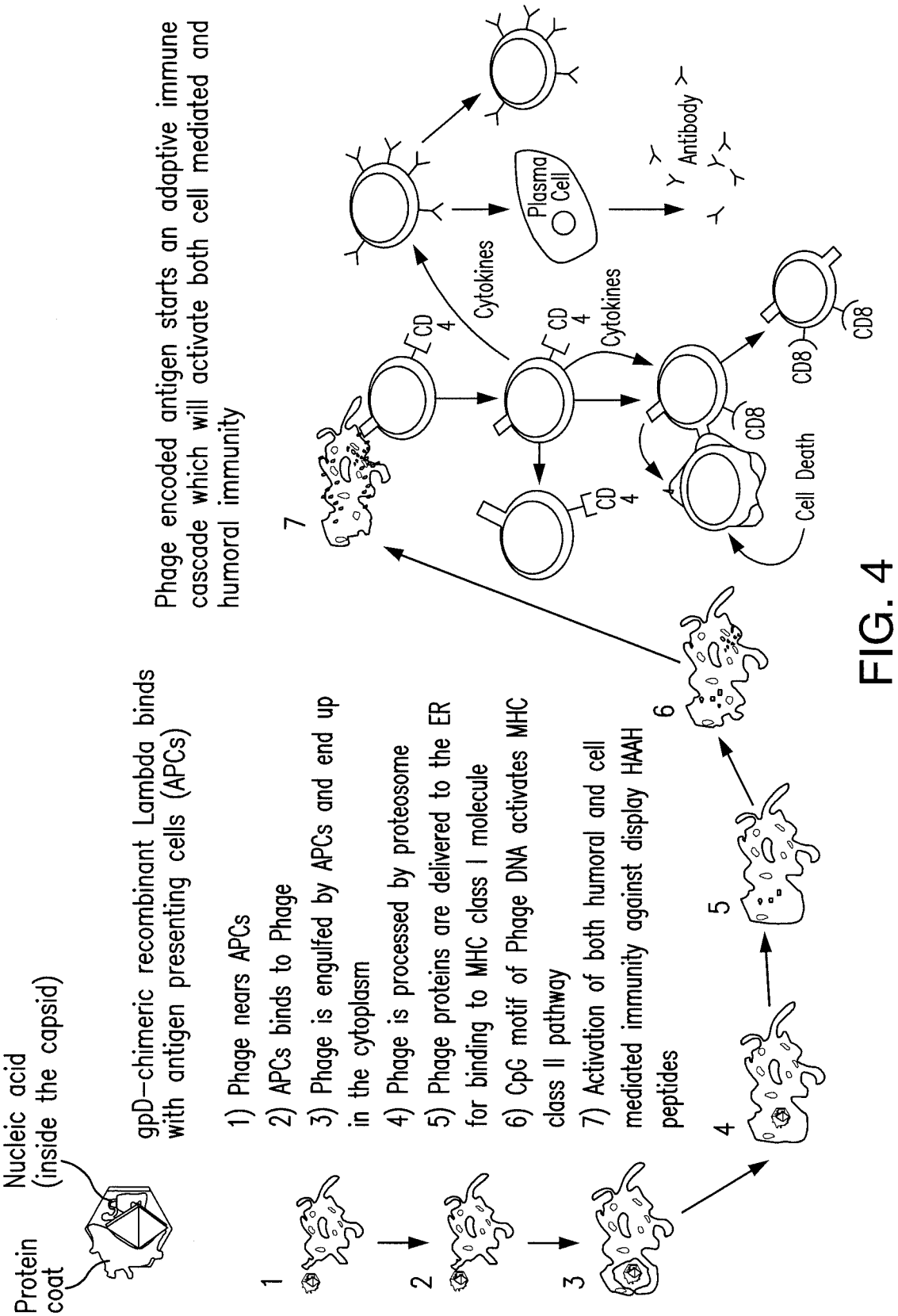

Protein coat
Nucleic acid (inside the capsid)
gpD–chimeric recombinant Lambda binds with antigen presenting cells (APCs)
1) Phage nears APCs
2) APCs binds to Phage
3) Phage is engulfed by APCs and end up in the cytoplasm
4) Phage is processed by proteosome
5) Phage proteins are delivered to the ER for binding to MHC class I molecule
6) CpG motif of Phage DNA activates MHC class II pathway
7) Activation of both humoral and cell mediated immunity against display HAAH peptides
Phage encoded antigen starts an adaptive immune cascade which will activate both cell mediated and humoral immunity
1
2
3
4
5
6
7
CD 4
Cytokines
Plasma Cell
Antibody
CD8
Cell Death

FIG. 4

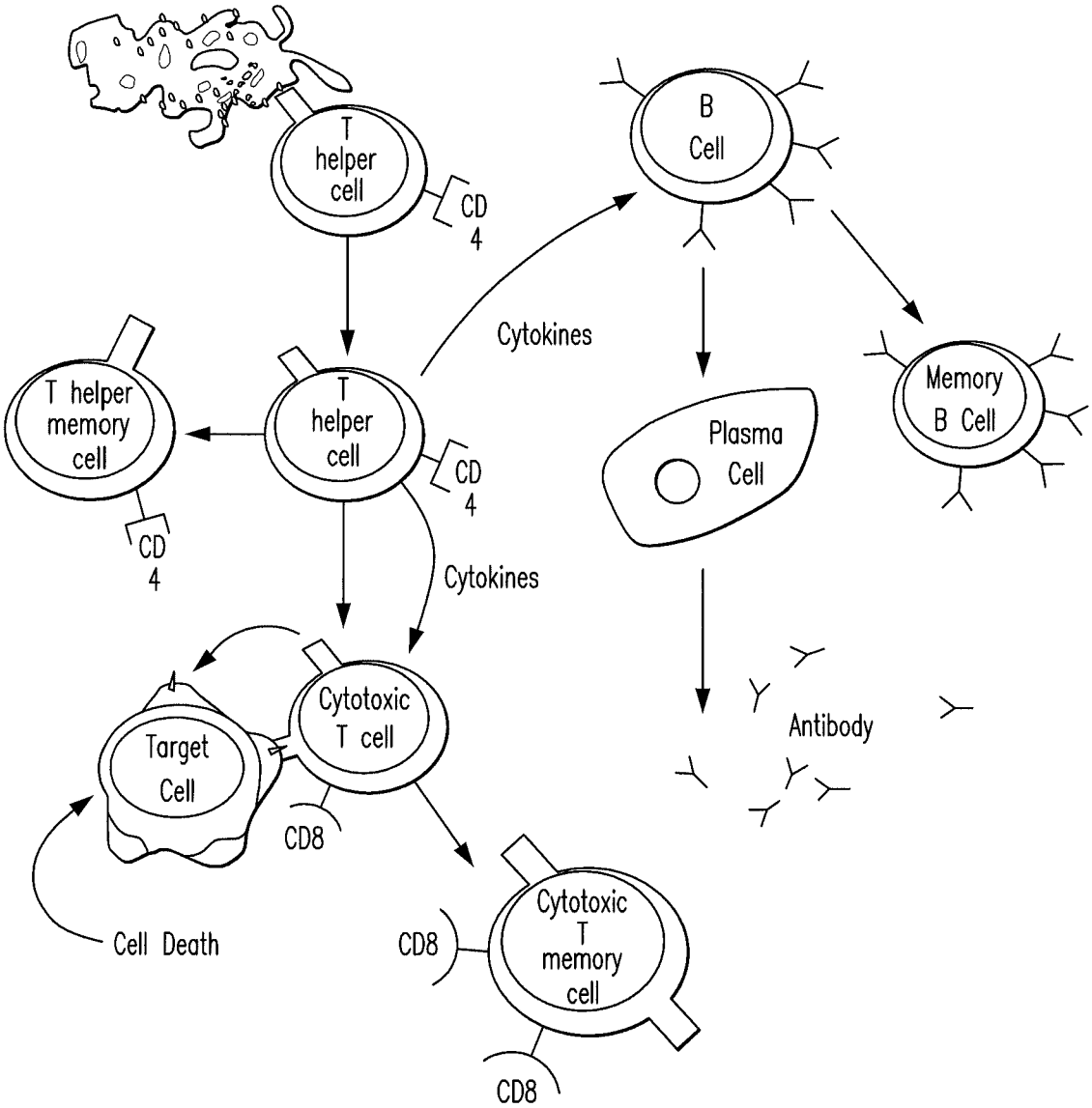

T helper cell
CD 4
B Cell
Cytokines
T helper memory cell
T helper cell
CD 4
Plasma Cell
Memory B Cell
CD 4
Cytokines
Cytotoxic T cell
Target Cell
Antibody
CD8
Cell Death
CD8
Cytotoxic T memory cell
CD8

FIG. 5

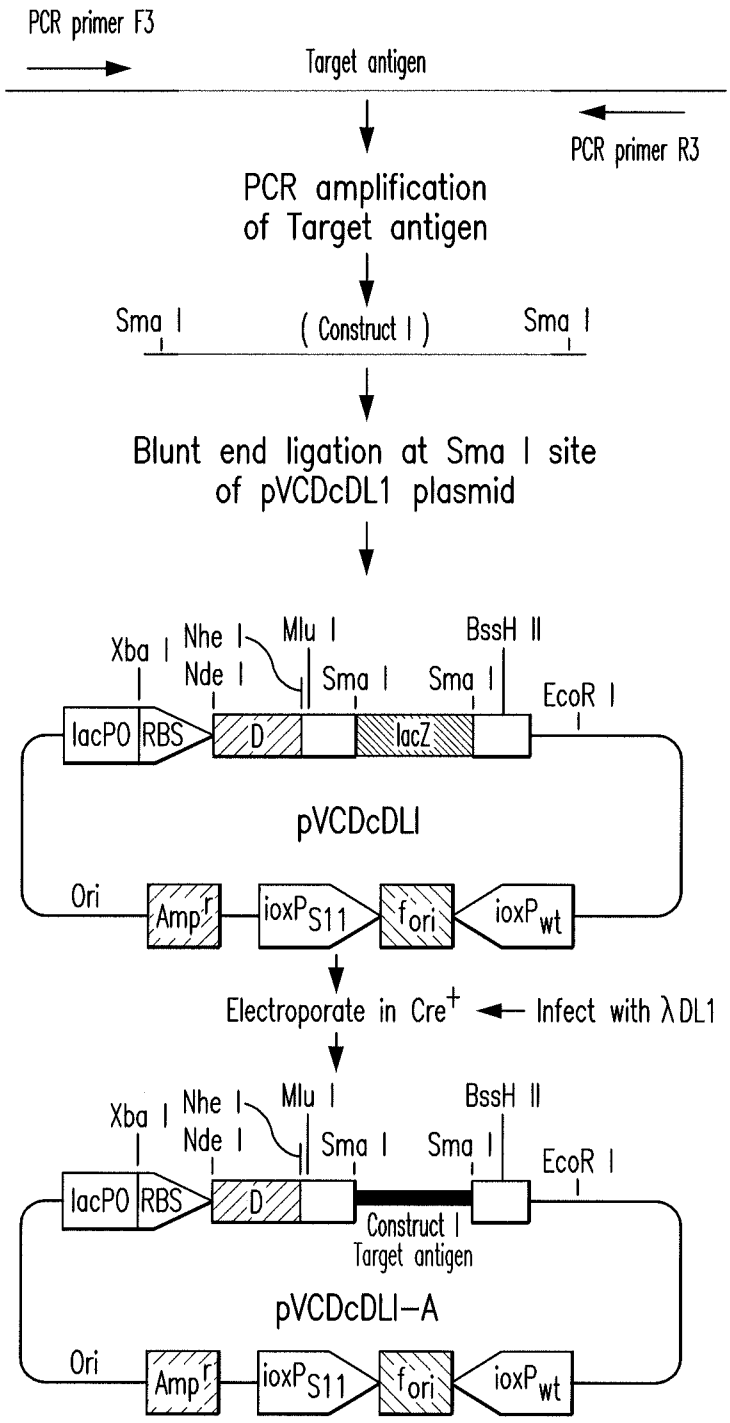

PCR primer F3
Target antigen
PCR primer R3
PCR amplification of Target antigen
Sma I
(Construct I)
Sma I
Blunt end ligation at Sma I site of pVCDcDL1 plasmid
Xba I
Nhe I
Nde I
Mlu I
Sma I
Sma I
BssH II
EcoR I
lacPO
RBS
D
lacZ
pVCDcDLI
Ori
Amp^r
ioxP S11
f ori
ioxP wt
Electroporate in Cre+
Infect with λDL1
Construct I
Target antigen
pVCDcDLI-A

FIG. 6A

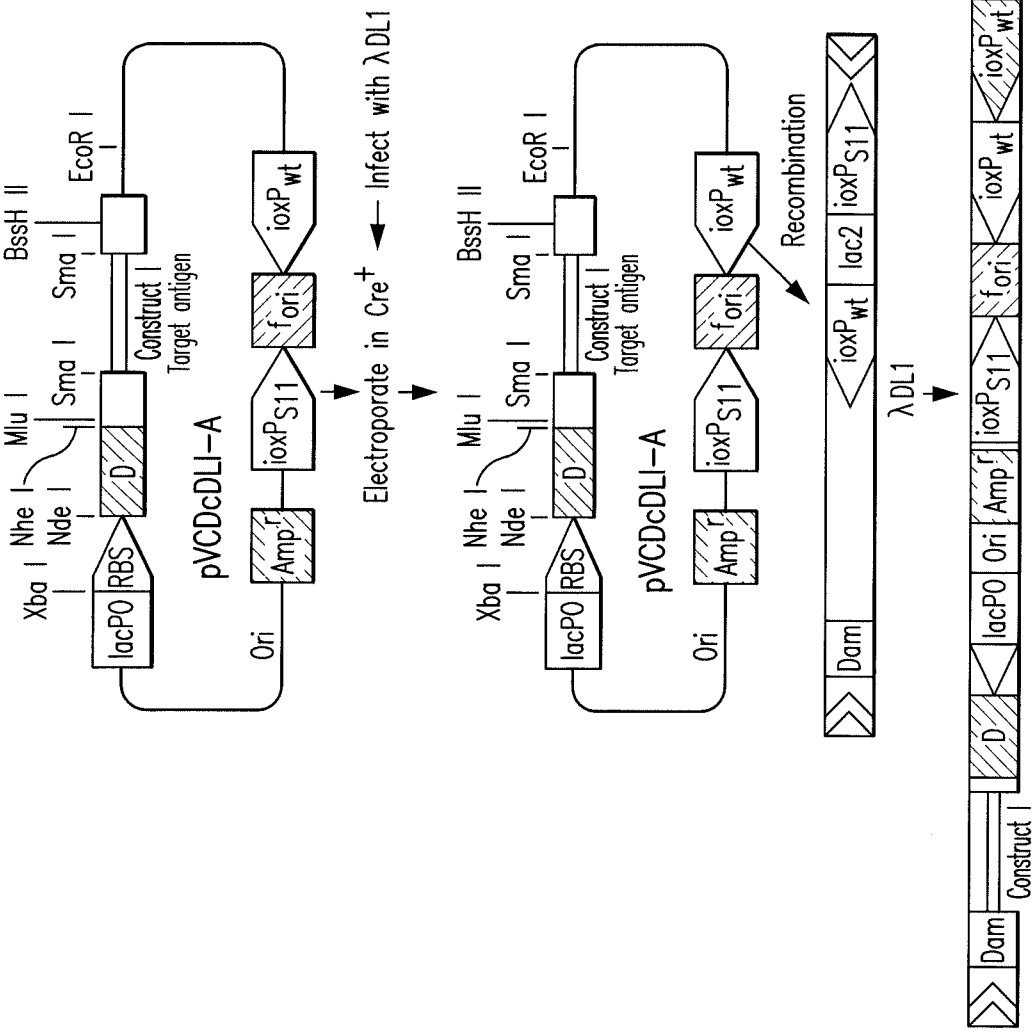

Xba I
Nhe I
Nde I
Mlu I
Sma I
BssH II
Sma I
EcoR I
lacPO
RBS
D
Construct I
Target antigen
pVCDcDLI-A
Ori
Amp r
ioxP S11
f ori
ioxP wt
Electroporate in Cre+
Infect with λDL1
Recombination
Dam
λDL1
lac2
Recombinant Phage for Vaccine
[High-density functional display of antigens as gpD fusion on Lambda head]

FIG. 6B

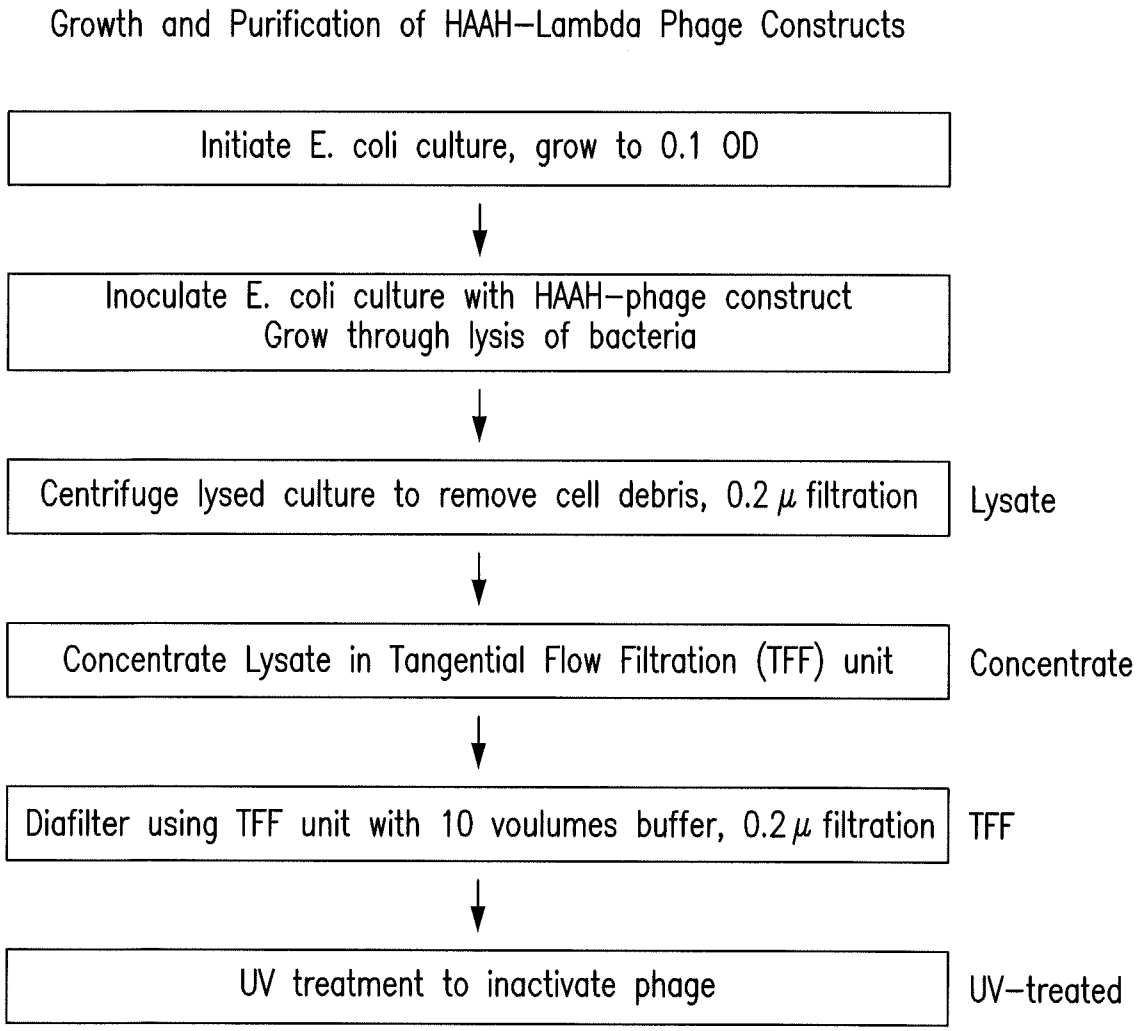

Growth and Purification of HAAH–Lambda Phage Constructs
Initiate E. coli culture, grow to 0.1 OD
Inoculate E. coli culture with HAAH–phage construct
Grow through lysis of bacteria
Centrifuge lysed culture to remove cell debris, 0.2 μ filtration
Lysate
Concentrate Lysate in Tangential Flow Filtration (TFF) unit
Concentrate
Diafilter using TFF unit with 10 voulumes buffer, 0.2 μ filtration
TFF
UV treatment to inactivate phage
UV–treated

FIG. 7

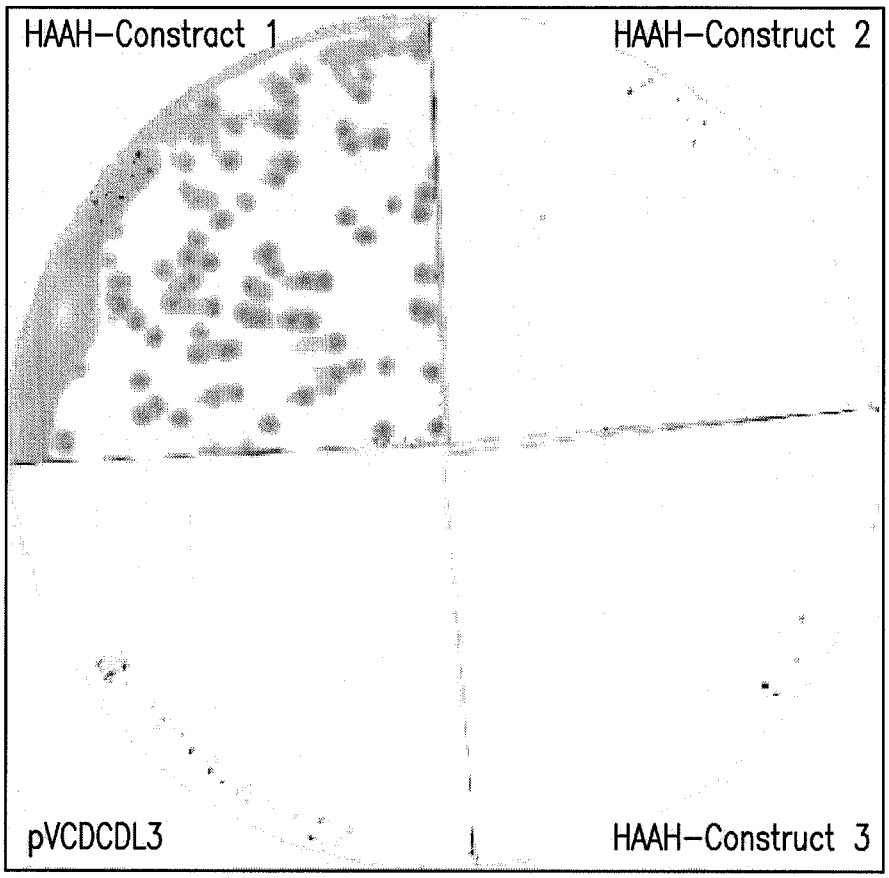

HAAH-Constract 1
HAAH-Construct 2
pVCDCDL3
HAAH-Construct 3

FIG. 8

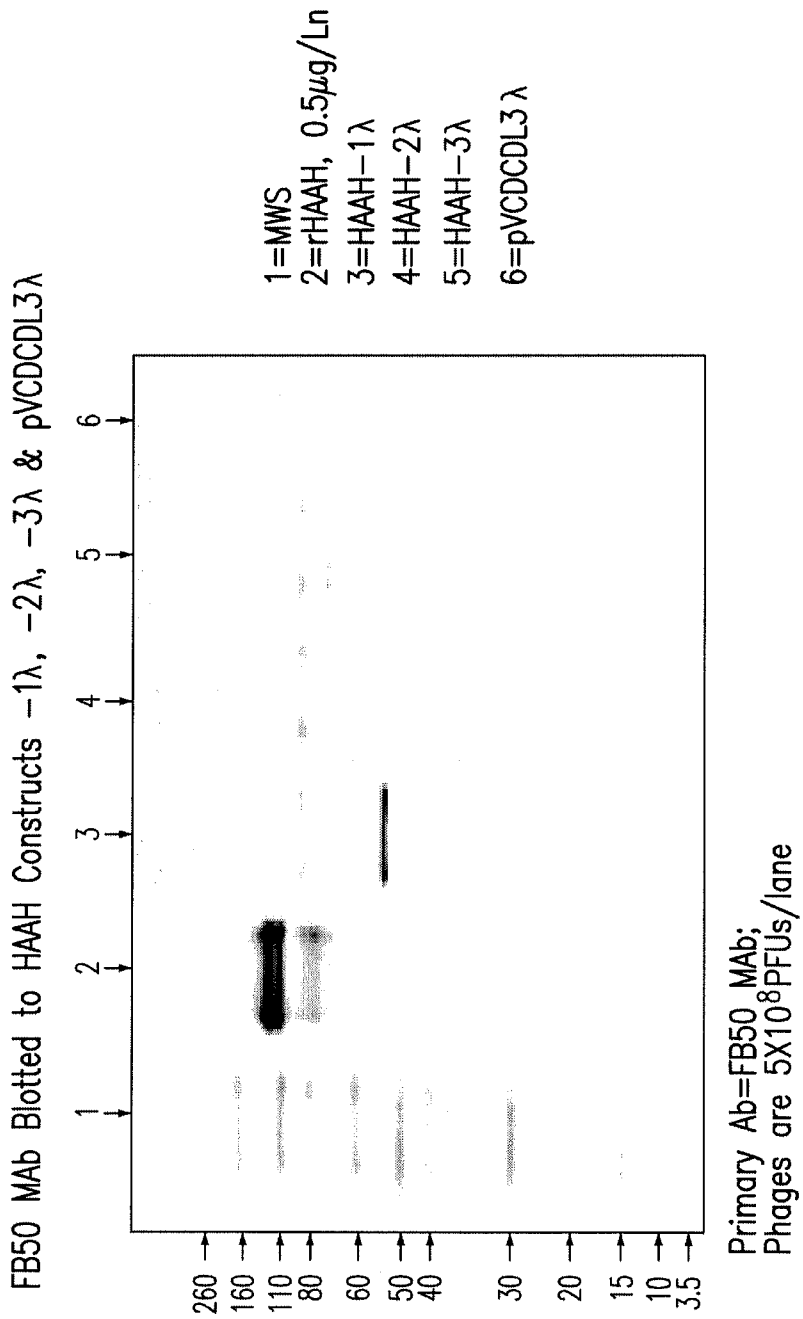

FB50 MAb Blotted to HAAH Constructs −1λ, −2λ, −3λ & pVCDCDL3λ
1=MWS
2=rHAAH, 0.5μg/Ln
3=HAAH−1λ
4=HAAH−2λ
5=HAAH−3λ
6=pVCDCDL3λ
1
2
3
4
5
6
260
160
110
80
60
50
40
30
20
15
10
3.5
Primary Ab=FB50 MAb;
Phages are 5X10^8PFUs/lane

FIG. 9

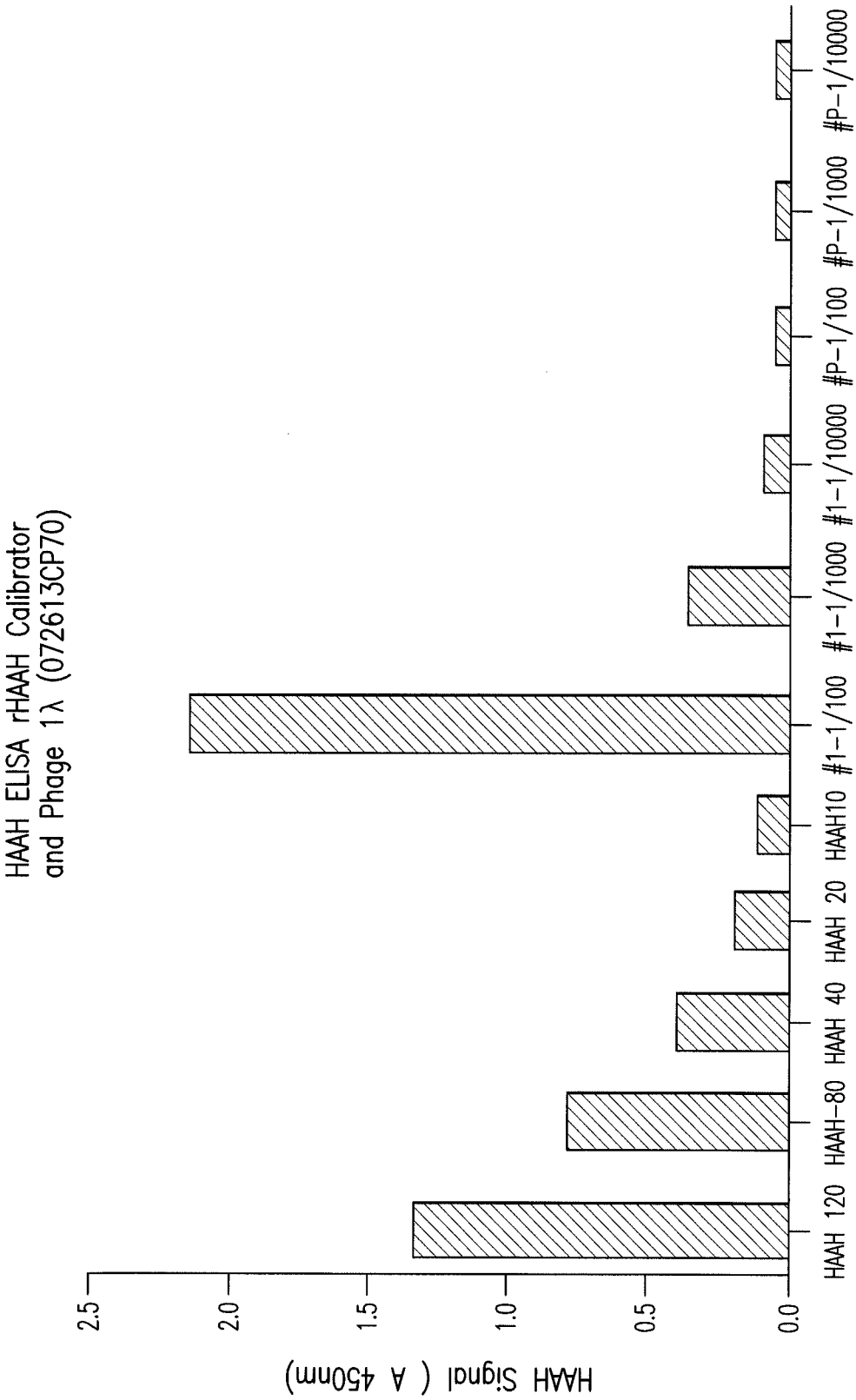

HAAH ELISA rHAAH Calibrator
and Phage 1λ (072613CP70)
HAAH Signal (A 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
HAAH 120
HAAH-80
HAAH 40
HAAH 20
HAAH10
#1-1/100
#1-1/1000
#1-1/10000
#P-1/100
#P-1/1000
#P-1/10000

FIG. 10

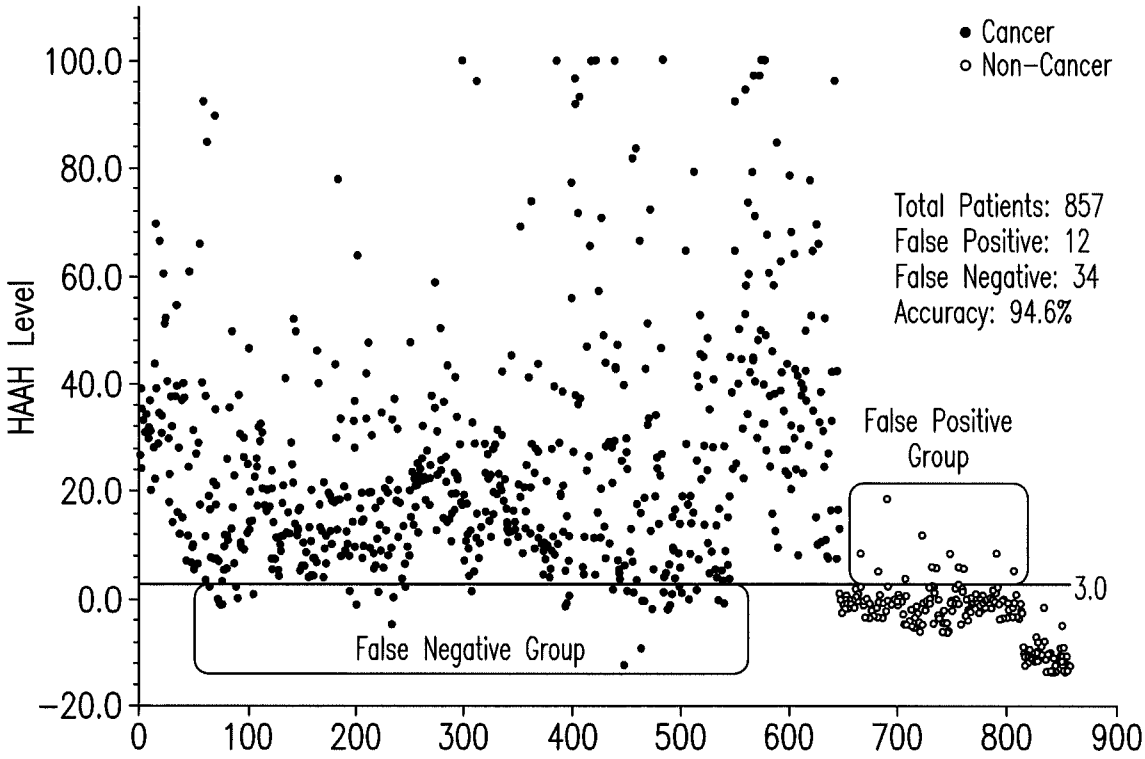

Cancer
Non-Cancer
Total Patients: 857
False Positive: 12
False Negative: 34
Accuracy: 94.6%
False Positive Group
False Negative Group
3.0
HAAH Level
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
0
100
200
300
400
500
600
700
800
900

FIG. 11

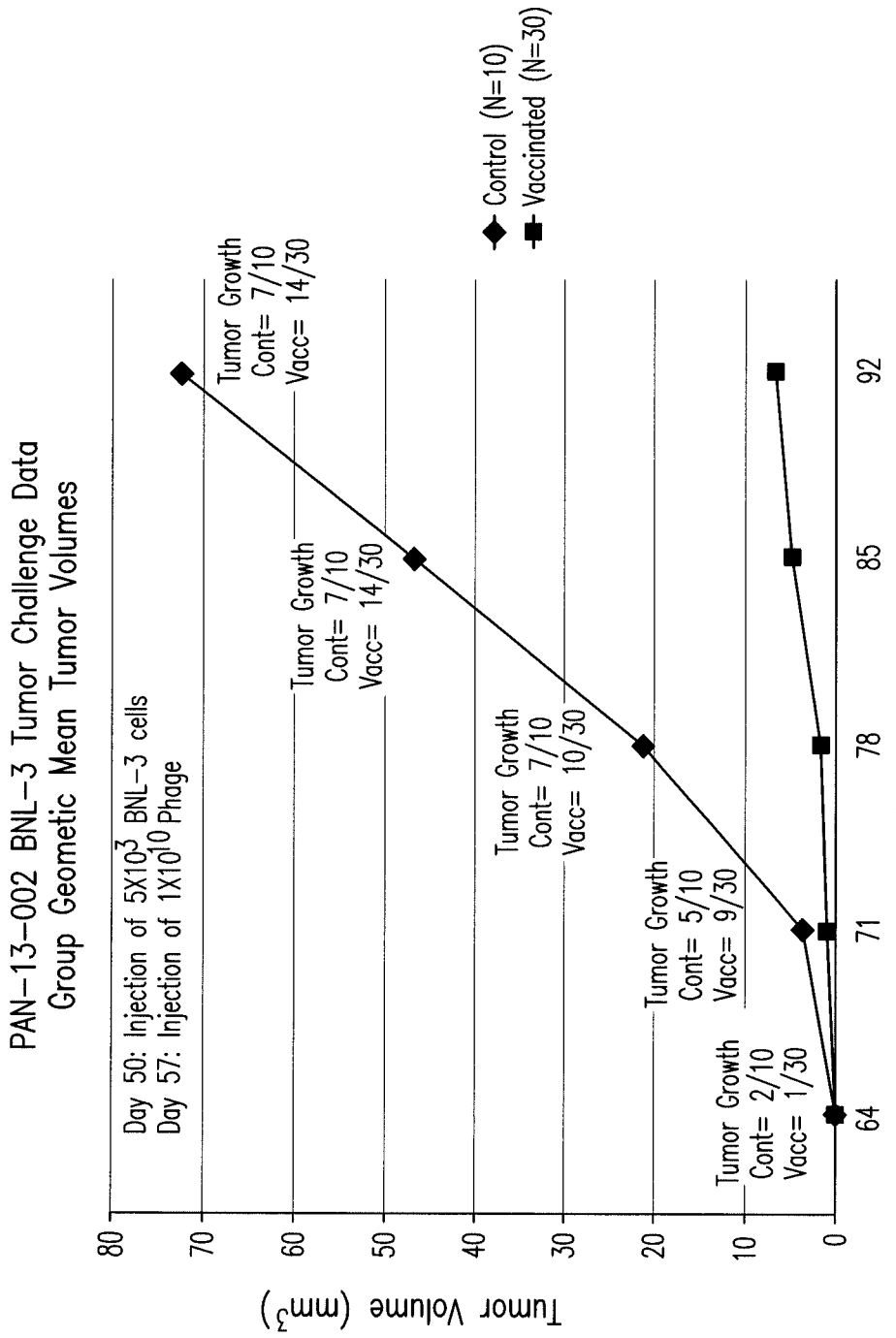

PAN-13-002 BNL-3 Tumor Challenge Data
Group Geometric Mean Tumor Volumes
Day 50: Injection of 5X10^3 BNL-3 cells
Day 57: Injection of 1X10^10 Phage
Control (N=10)
Vaccinated (N=30)
Tumor Growth
Cont= 7/10
Vacc= 14/30
Tumor Growth
Cont= 7/10
Vacc= 14/30
Tumor Growth
Cont= 7/10
Vacc= 10/30
Tumor Growth
Cont= 5/10
Vacc= 9/30
Tumor Growth
Cont= 2/10
Vacc= 1/30
Tumor Volume (mm^3)
80
70
60
50
40
30
20
10
0
64
71
78
85
92
Study Day

FIG. 12

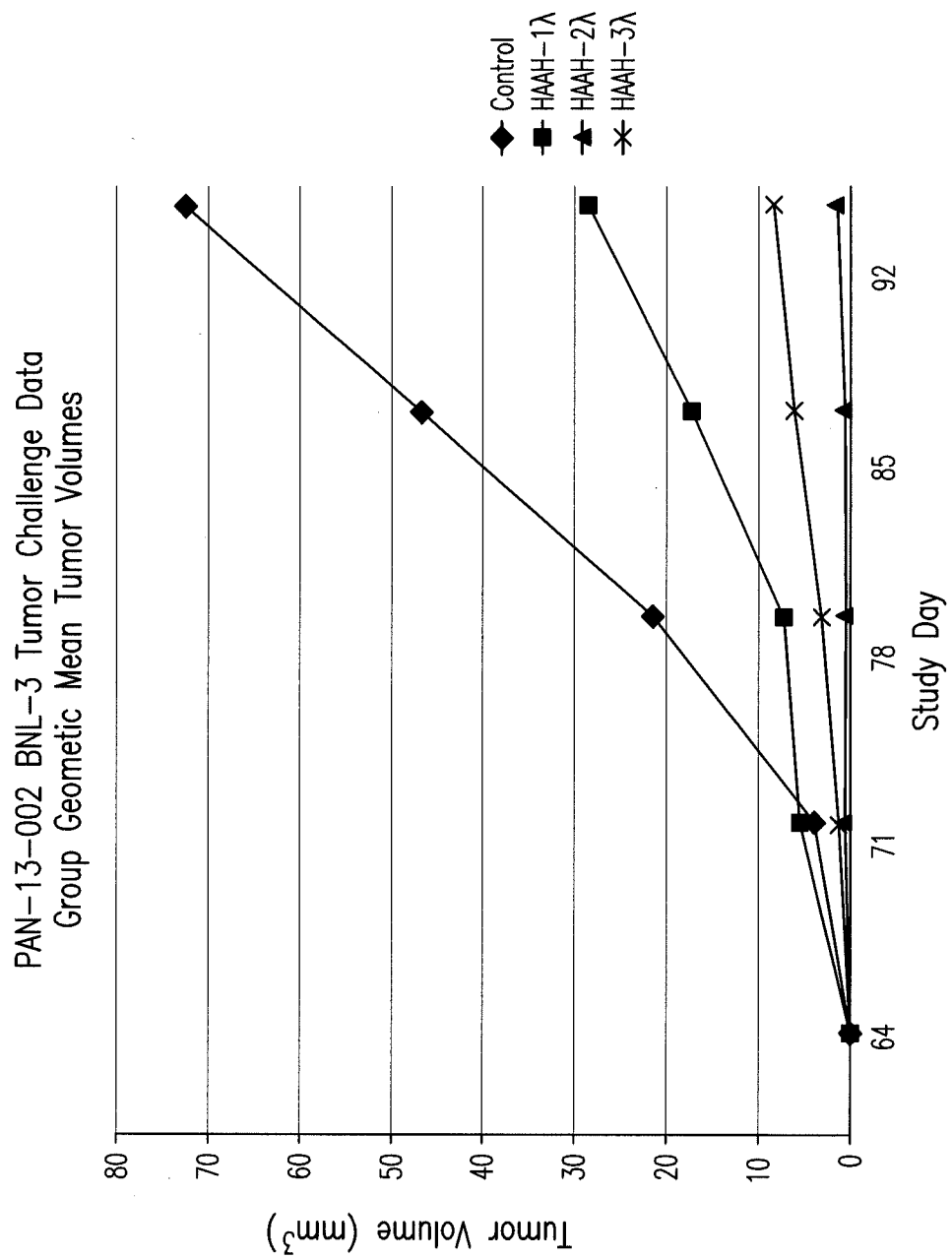

PAN-13-002 BNL-3 Tumor Challenge Data
Group Geometic Mean Tumor Volumes
Control
HAAH-1λ
HAAH-2λ
HAAH-3λ
Tumor Volume (mm³)
0
10
20
30
40
50
60
70
80
64
71
78
85
92
Study Day

FIG. 13

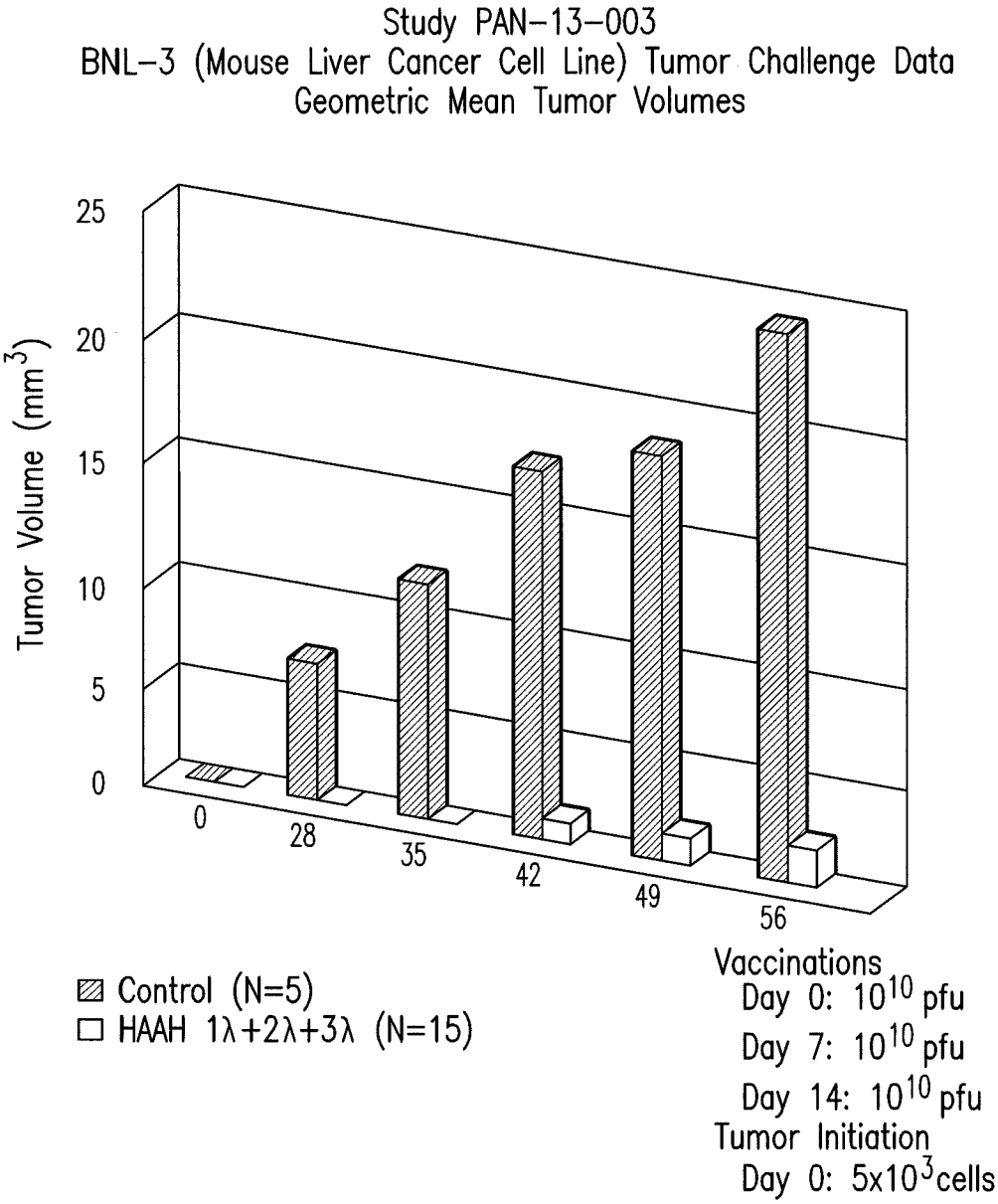
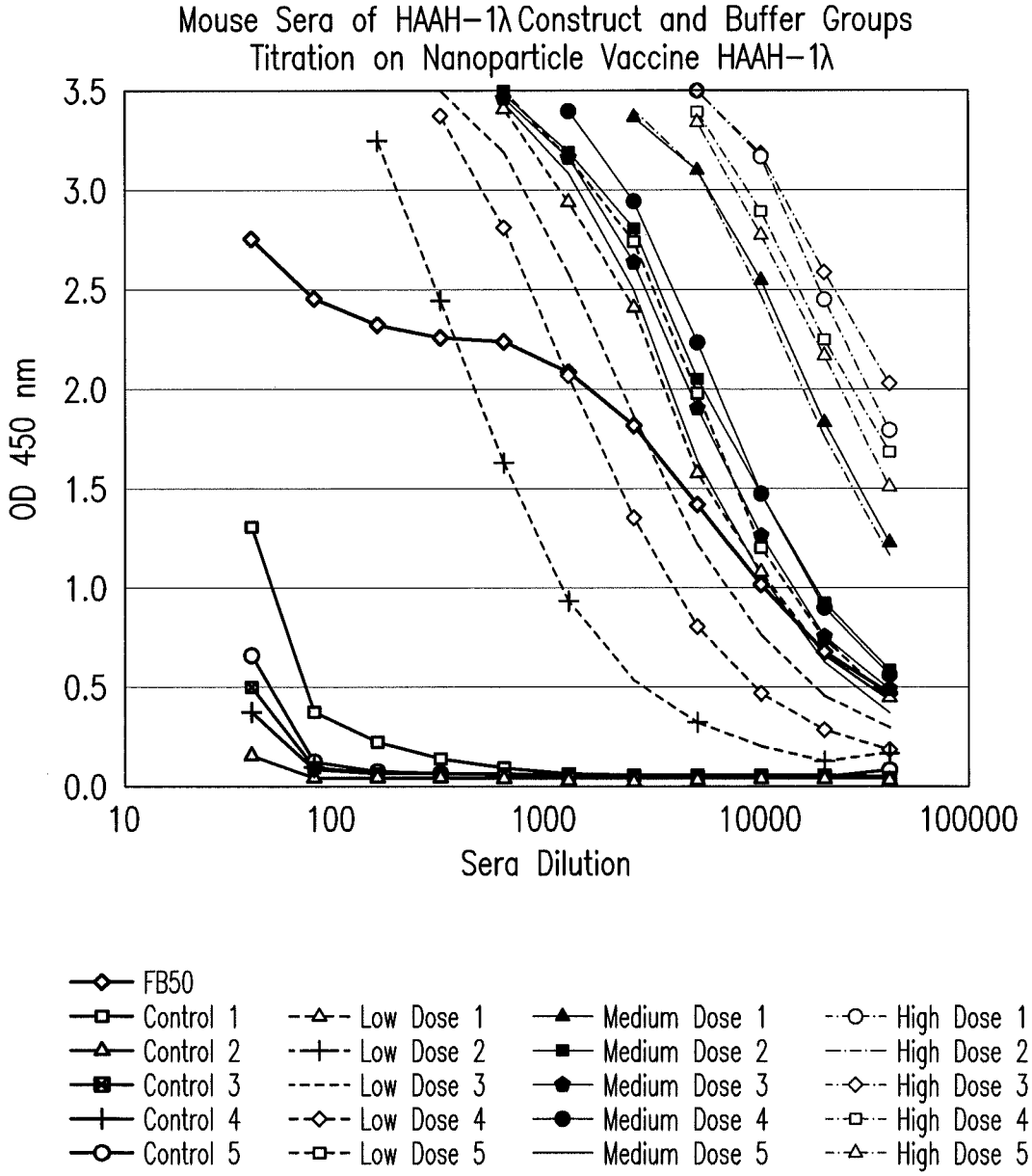
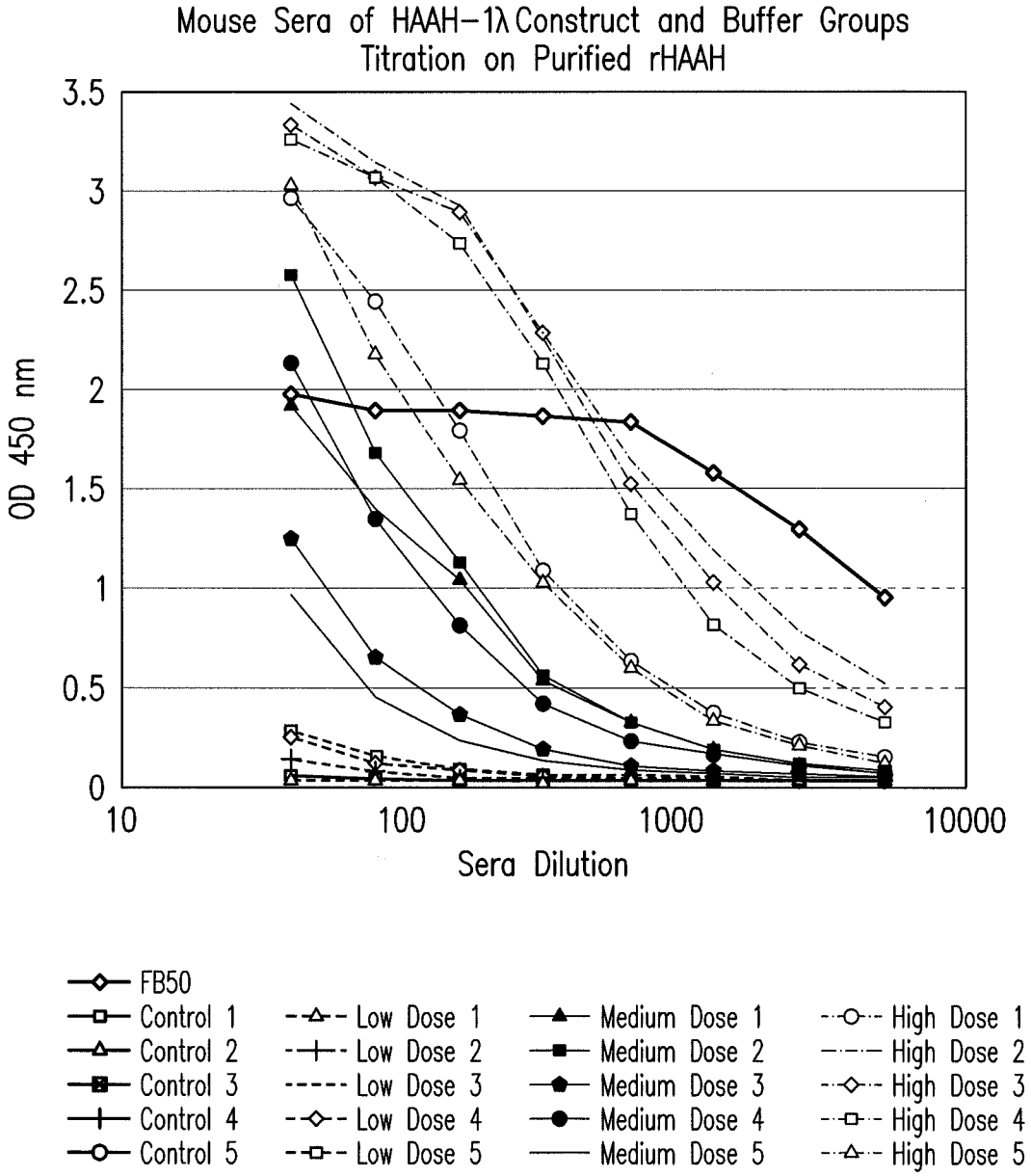

Study PAN-13-003
BNL-3 (Mouse Liver Cancer Cell Line) Tumor Challenge Data
Geometric Mean Tumor Volumes
Tumor Volume (mm3)
25
20
15
10
5
0
0
28
35
42
49
56
Control (N=5)
HAAH 1λ+2λ+3λ (N=15)
Vaccinations
Day 0: 10^10 pfu
Day 7: 10^10 pfu
Day 14: 10^10 pfu
Tumor Initiation
Day 0: 5x10^3cells

FIG. 14



Sera Titration: 2-fold serial dilutions starting at 1/40
Control = Buffer Control
Low Dose = HAAH-1λ, 5X10E7 PFUs
Medium Dose = HAAH-1λ, 5X10E8 PFUs
High Dose = HAAH-1λ, 5X10E9 PFUs
FB50 monocional antibody titrated starting at 5.0μg/mL

FIG. 15



Sera Titration: 2-fold dilutions starting at 1/40
Control = Buffer Control
Low Dose = HAAH-1λ, 5X10E7 PFUs
Medium Dose = HAAH-1λ, 5X10E8 PFUs
High Dose = HAAH-1λ, 5X10E9 PFUs
FB50 titrated starting at 312.5ng/mL

FIG. 16

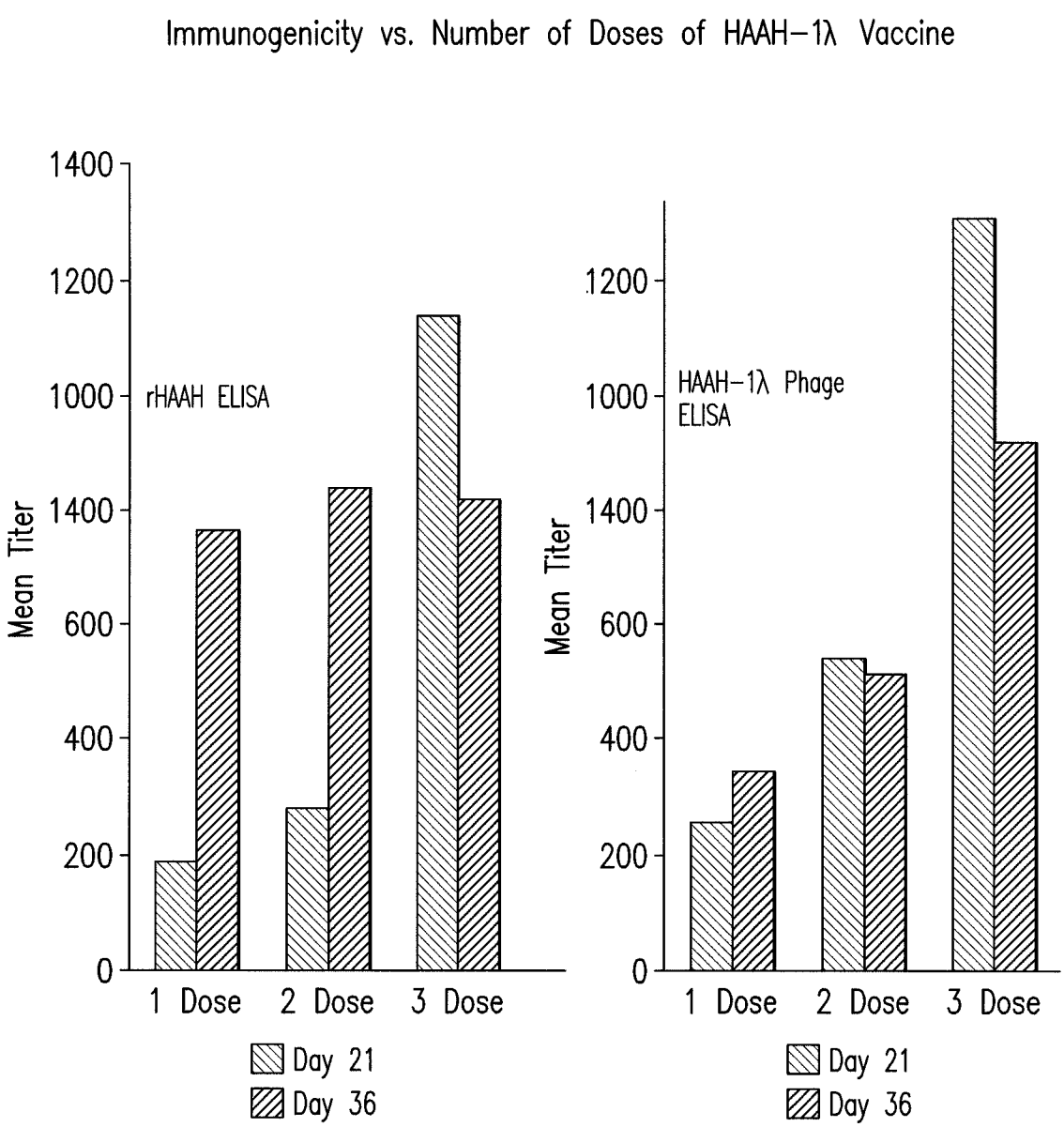

Immunogenicity vs. Number of Doses of HAAH-1λ Vaccine
rHAAH ELISA
HAAH-1λ Phage ELISA
Mean Titer
1400
1200
1000
1400
600
400
200
0
1 Dose
2 Dose
3 Dose
Day 21
Day 36

FIG. 17

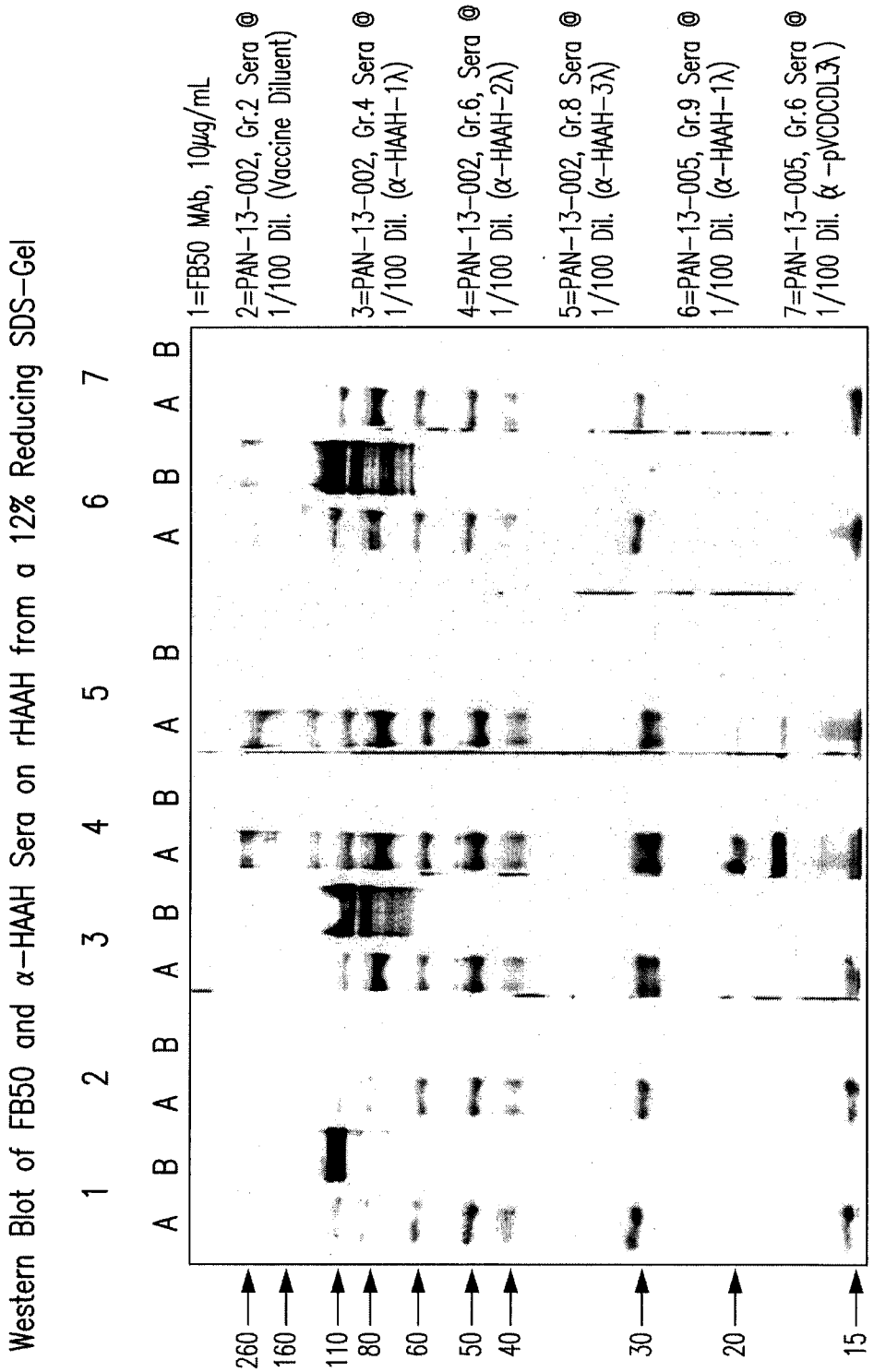

Western Blot of FB50 and α-HAAH Sera on rHAAH from a 12% Reducing SDS-Gel
1 2 3 4 5 6 7
A B A B A B A B A B A B A B
1=FB50 MAb, 10μg/mL
2=PAN-13-002, Gr.2 Sera @ 1/100 Dil. (Vaccine Diluent)
3=PAN-13-002, Gr.4 Sera @ 1/100 Dil. (α-HAAH-1λ)
4=PAN-13-002, Gr.6, Sera @ 1/100 Dil. (α-HAAH-2λ)
5=PAN-13-002, Gr.8 Sera @ 1/100 Dil. (α-HAAH-3λ)
6=PAN-13-005, Gr.9 Sera @ 1/100 Dil. (α-HAAH-1λ)
7=PAN-13-005, Gr.6 Sera @ 1/100 Dil. (α-pVCDCDL3λ)
260
160
110
80
60
50
40
30
20
15
A=MWS
B=rHAAH at 0.5μg/lane

FIG. 18

Map HAAH-Construct I

DRAMAQRKNAKSSGNSSSSGSGSGSTSAGSSSPGARRETKHGGHKNGRKG 50
GLSGTSFFTWFMVIALLGVWTSVAVVWFDLVDYEEVLGKLGIYDADGDGD 100
FDVDDAKVLLGLKERSTSEPAVPPEEAEPHTEPEEQVPVEAEPQNIEDEA 150
KEQIQSLLHEMVHAEHVEGEDLQQEDGPTGEPQQEDDEFLMATDVDDRFE 200
TLEPEVSHEETEHSYHVEETVSQDCNQDMEEMMSEQENPDSSEPVVEDER 250
LHHDTDDVTYQVYEEQAVYEPLENEGIEITEVTAPPEDNPVEDSQVIVEE 300
VSIFPVEEQQEVPP

Map HAAH-Construct II

LDA 350
AEKLRKRGKIEEAVNAFKELVRKYPQSPRARYGKAQCEDDLAEKRRSNEV 400
LRGAIETYQEVASLPDVPADLLKLSLKRRSDRQQFLGHMRGSLLTLQRLV 450
QLFPNDTSLKNDLGVGYLLIGDNDNAKKVYEEVLSVTPNDGFAKVHYGFI 500
LKAQNKIAESIPYLKEGIESGDP

Map HAAH-Construct III

GTDDGRFYFHLGDAMQRVGNKEAYKWY 550
ELGHKRGHFASVWQRSLYNVNGLKAQPWWTPKETGYTELVKSLERNWKLI 600
RDEGLAVMDKAKGLFLPEDENLREKGDWSQFTLWQQGRRNENACKGAPKT 650
CTLLEKFPETTGCRRGQIKYSIMHPGTHVWPHTGPTNCRLRMHLGLVIPK 700
EGCKIRCANETRTWEEGKVLIFDDSFEHEVWQDASSFRLIFIVDVWHPEL 750
TPQQRRSLPAI*HEFMQAWET

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/30551

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 7/00, 7/01; A01N 63/00 (2014.01)
USPC - 424/93.6, 93.1, 184.1; 435/235.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classification(s): C12N 7/00, 7/01; A01N 63/00 (2014.01)
USPC Classification(s): 424/93.6, 93.1, 184.1; 435/235.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google/Google Scholar; PubMed; ProQuest; bacteriophage, 'Asparty[Asparaginyl] -β-hydroxylase', HAAH, 'Amino acid sequence', gpD

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y — A	WO 2007/076101 A2 (MERRIL, C et al.) July 05, 2007; page 6, paragraphs 4-5; page 7, paragraph 2; page 12, paragraph 2; page 13, paragraph 2; page 16, paragraphs 1-2; page 17, paragraphs 3-4; Claims 1, 5; figures 4, 8	1, 5-7, 19 — 3, 8, 10, 12, 17, 18 — 2, 4, 9, 11, 14, 16, 20
Y — A	US 2005/0123545 A1 (WANDS, J et al.) June 09, 2005; abstract; Table 1	3, 10, 13, 15, 17, 18 — 2, 4, 9, 11, 14, 16, 20
Y	YANG, F et al. Novel Fold And Capsid-Binding Properties Of The λ-phage Display Platform Protein gpD. Nature Structural Biology. March 2000, Vol. 7, pp 230-237; figure 3b	8, 10, 12
Y	US 2005/0226892 A1 (RAO, VB) October 13, 2005; paragraphs [0038], [0057]	13, 15, 17, 18
A	DINCHUK, JE et al. Aspartyl b-Hydroxylase (Asph) and an Evolutionarily Conserved Isoform of Asph Missing the Catalytic Domain Share Exons with Junctin. The Journal of Biological Chemistry. 23 August 2000, Vol. 275, pp 39543–39554; figure 2; DOI10.1074/jbc.M006753200.	2, 4, 9, 11, 14, 16, 20
A	LAVAISSIERE, L et al. Overexpression Of Human Aspartyl(asparaginyl)beta-hydroxylase In Hepatocellular Carcinoma and Cholangiocarcinoma. NCBI GenBank Accession No. AAB50779.1, aspartyl(asparaginyl)beta-hydroxylase [Homo sapiens]. Submitted 1996; downloaded from the internet <http://www.ncbi.nlm.nih.gov/protein/1911652?report=genbank&log$=protalign&blast_rank=1&RID=XGP8TSN8015> on 29 July 2014, pp 1-2.	2, 4, 9, 11, 14, 16, 20

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