(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2018 (25.01.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/018041 A1

(51) International Patent Classification:
A61K 39/112 (2006.01) *C07K 14/005* (2006.01)
A61K 39/12 (2006.01) *C12N 1/21* (2006.01)

(21) International Application Number:
PCT/US2017/043511

(22) International Filing Date:
24 July 2017 (24.07.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/365,549 22 July 2016 (22.07.2016) US
62/467,340 06 March 2017 (06.03.2017) US

(71) Applicant: **UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INCORPORATED** [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventor: **CURTISS, Roy, III**; 2312 N.W. 23rd Terrace, Gainesville, FL 32605-2807 (US).

(74) Agent: **EISENSCHENK, Frank, C.** et al.; Saliwanchik, Lloyd & Eisenschenk, P.O. Box 142950, Gainesville, FL 32614-2950 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2018/018041 A1

(54) Title: DESIGN OF PROTECTIVE ANTI-ZIKV VACCINE WITHOUT INDUCING CROSS-REACTIONS WITH DENGUE

(57) Abstract: The subject application provides a genetically modified recombinant facultative intracellular invasive bacterial pathogen (RFIIBP) or a recombinant attenuated *Salmonella* vaccine (RASV) strains encoding Zika virus (ZIKV) antigens. These strains exhibit high immunogenicity, complete safety, and attenuation, and are unable to persist or be shed in an infective or viable form. These RFIIBPs and RASVs also exhibit regulated delayed attenuation *in vivo*, regulated delayed *in vivo* synthesis of protective ZIKV antigens. Methods of inducing mucosal, systemic and cellular immunities in hosts are also provided and antibodies produced using the disclosed RFIIBPs and RASVs can comprise neutralizing antibodies against ZIKV that do not crossreact with Dengue virus (DENV).

DESIGN OF PROTECTIVE ANTI-ZIKV VACCINE WITHOUT INDUCING CROSS-REACTIONS WITH DENGUE

DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 62/365,549, filed July 22, 2017, and Serial No. 62/467,340, filed March 6, 2017, the disclosures of which are hereby incorporated by reference in their entirety, including all figures, tables and amino acid or nucleic acid sequences.

The Sequence Listing for this application is labeled "Seq-List.txt" which was created on July 24, 2017 and is 88 KB. The entire content of the sequence listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Zika virus (ZIKV) infections are often associated with the adverse sequelae microcephaly, Guillian-Barre syndrome and, likely, other nervous system disorders that may be associated with prior exposure to dengue virus (DENV). Since ZIKV is transmitted by daytime active *Aedes aegypti* mosquitoes that are prevalent in the southern US, it is of critical importance to design and develop a safe efficacious vaccine to prevent ZIKV infection and especially to not induce immune responses that would be cross reactive with any of the four dengue virus types.

The subject application provides genetically modified recombinant facultative intracellular invasive bacterial pathogens that synthesize ZIKV protein antigens and are capable of: inducing mucosal immunity; and/or inducing CD4, and especially CD8 immune responses that should kill virus-infected cells. The genetically modified recombinant facultative intracellular invasive bacterial pathogens can be manufactured by fermentation technology to yield 1000-times more vaccine doses per liter of culture than can be produced using traditional DNA vaccine technologies and have the advantage of preservation by lyophilization in a thermostable form that obviates need for a cold chain. The lyophilized genetically modified recombinant facultative intracellular invasive bacterial pathogens can be reconstituted at a time and place for needle-free oral administration (not requiring highly trained medical care givers) for inducing immunity to ZIKV protein antigens.

In specific embodiments, the subject application provides newly developed innovative recombinant attenuated *Salmonella* vaccine (RASV) strains to deliver multiple ZIKV antigens (or other antigens) as a cost-effective vaccine to induce mucosal and systemic protective immunity. In addition to the advantages and characteristics discussed above, the disclosed RASV strains are attenuated, provide high immunogenicity and exhibit a desirable safety profile.

BRIEF SUMMARY OF THE INVENTION

It has been found that antibodies neutralizing dengue virus (DENV) react with ZIKV and disease symptoms after ZIKV infection are much more severe in animals previously infected with DENV or immunized with a DENV vaccine. This strongly suggests that cross reactivity of these two flaviviruses has the potential to complicate the development of a safe, efficacious vaccine to prevent or reduce either ZIKV or DENV infections. The cross-reactivity of neutralizing antibodies may also explain why the severe sequelae associated with ZIKV infections noted above are most often observed in countries with widespread previous exposure to DENV infections. It thus becomes important to develop vaccines that are safe and efficacious in conferring protection against flaviviruses, such as ZIKV and DENV, and that do not induce cross-reactive immune responses to each other or to other flaviviruses.

The subject application provides genetically modified recombinant facultative intracellular invasive bacterial pathogens (RFIIBPs), for example recombinant attenuated *Salmonella* vaccine (RASV) strains, with high immunogenicity, complete safety, and attenuation, and which also are unable to persist or be shed in an infective or viable form. These genetically modified recombinant facultative intracellular invasive bacterial pathogens also exhibit regulated delayed attenuation *in vivo* and regulated delayed *in vivo* synthesis of protective antigens. These antigens can be encoded by codon-optimized DNA sequences (in some embodiments, see, for example, Figures 3-6), and the genetically modified recombinant facultative intracellular invasive bacterial pathogens exhibit regulated delayed lysis *in vivo*.

The disclosed genetically modified recombinant facultative intracellular invasive bacterial pathogens system allows vaccines to be grown under conditions that enable them to display, after oral immunization, the capabilities of a wild-type strain. These RASV strains survive host defense stresses and efficiently colonize effector lymphoid tissues before manifesting attenuation (which precludes disease symptoms) and synthesizing protein antigens or to deliver DNA vaccines encoding antigens that induce protective immune

responses. This system causes amplification of protective antigen production *in vivo* by the immunized host to induce mucosal and systemic antibody responses and also induces mucosal and systemic cellular immunity. The disclosed genetically modified recombinant facultative intracellular invasive bacterial pathogens system allows for large-scale, efficient, and economical production of genetically modified recombinant facultative intracellular invasive bacterial pathogens vaccines in a thermostable form for oral administration. These RASV vaccines are designed to generate safe, protective immunity against ZIKV and could also be used to design vaccines against other flaviviruses and reduce or eliminate the potential that vaccination against one flavivirus will engender immunity that will exacerbate disease caused by infection with, or vaccination against, another flavivirus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1I. Plasmid maps. Lysis vector pYA4763 (1A). DNA vaccine vector designs are illustrated in Figures 1B (base vector), 1G (base vector containing nucleic acid sequence encoding non-codon optimized envelope (E) protein) and 1H (base vector containing nucleic acid sequence encoding codon optimized envelope (E) protein and amino acid substitutions at ASN154 and ASN481). Vector designs for expressing vaccine antigens (antigen-encoding lysis vectors) in the disclosed RFIIBPs and RASVs are illustrated in Figures 1C (base vector with modified Shine Dalgarno sequences), 1D (base vector), 1E (base vector of Fig. 1D containing nucleic acid sequence encoding codon optimized envelope (E) protein) and 1F (base vector of Fig. 1D containing nucleic acid sequence encoding codon optimized envelope (E) protein and amino acid substitutions at ASN154 and ASN481) and 1I (base vector containing nucleic acid sequence encoding a fusion protein of membrane precursor (prM) and NS5 (each of which is not codon optimized).

Figure 2. Phylogenetic tree of Zika virus isolates identified from Guatemala and Puerto Rico in December 2015 (indicated in boldface) compared with reference isolates obtained from GenBank. The isolates from Guatemala and Puerto Rico grouped with other Asian genotype viruses. The tree was derived by neighbor-joining methods (bootstrapped 1,000 times) using complete-genome sequences. Location, year identified, and GenBank strain identification for the viruses used in tree construction are shown. Scale bar indicates number of nucleotide substitutions per site. GenBank accession nos.: KU321639 (Brazil 2015 SPH2015), KJ776791 (French Polynesia H/PF/2013), KF383115 (Central African Republic ARB1362), KF383116 (Senegal 1968 ArD7117), KF383117 (Senegal 1997 ArD128000),

KF383118 (Senegal 2001 ArD157995), KF383119 (Senegal 2001 ArD158084), KF268948 (CAR 1979 ARB13565), KF268949 (CAR 1980 ARB15076), KF268950 (CAR 1976 ARB7701), EU545988 (Yap 2007), KF993678 (Thailand 2013 PLCal_ZV), JN860885 (Cambodia 2010 FSS13025), HQ234499 (Malaysia 1966 P6-740), HQ234501 (Senegal 1984 ArD41519), HQ234500 (Nigeria 1968 IbH 30656), LC002520 (Uganda 1947 MR766), KU501215 (Puerto Rico PRVABC59), KU501216 (Guatemala 8375), and KU501217 (Guatemala 103344) [published by Lanciotti RS, Lambert AJ, Holodniy M, Saavedra S, Signor Ldel C., 2016, Phylogeny of Zika Virus in Western Hemisphere, 2015. Emerg Infect Dis. 22(5):933-5)].

Figures 3-6. Native (upper sequence) and codon-optimized (lower) nucleic acid sequences encoding ZIKV antigens. Figure 3 illustrates capsid protein C (309 bp, (103 aa) with 22 aa codons optimized (29 bp changed)). Figure 4 depicts prM 288 bp ((96 aa) optimized and 279 bp (93 aa) original with 12 aa (18 bp) codon optimized and 3 N-terminal codons added in the optimized sequence). Figure 5 shows E protein (1518 bp (506 aa) in codon optimized and 1512 bp (504 aa) in original; codon for aa 156(154 in original) and 483 (481 in original) changed to code Q in place of N; 54 aa (72 bp) were codon optimized and 2 N-terminal codons added in the optimized sequence). Figure 6 illustrates NS5 (2718 bp (906 aa) in the codon optimized sequence and 2709 bp (903 aa) in original ZIKV sequence; 137 aa (196 bp) were codon optimized with 3 N-terminal codons added in the optimized sequence.

Figure 7. Sequence of pYA4545.

Figure 8. Plasmid maps for pYA4763 prM-E and pG8R17 prM-E. ZIKV prM and E proteins encoded by sequences with codons most frequently used in highly expressed *Salmonella* genes as given for the DNA sequences listed on line 2 in Figures 10A and 10B.

Figure 9. prM-E vector maps for pYA4763 prM-E* and pG8R17 prM-E*. ZIKV prM and E proteins encoded by sequences with codons most frequently used in the most highly expressed *Salmonella* genes as given for the DNA sequences listed on line 3 in Figures 10A and 10B with *Salmonella*-synthesized proteins retained in RASV cells for release during regulated delayed lysis (pYA4763-derived construct) or continually secreted due to fusion to the type 2 secretion system β-lactamase into the RASV periplasm to generate outer membrane vesicles and also continually released extracellularly to augment induction of immune responses prior to a release of a bolus of antigens at the time of regulated lysis in vivo (pG8R17-derived construct).

Figures 10A and 10B. Nucleotide and amino acid sequences for PrM (Figure 10A) and E protein (Figure 10B). First line – original codon sequences in ZIKV; second line – codons optimized for good expression in *Salmonella*; third line – codons selected to represent the codons most frequently used by the most highly expressed *Salmonella* genes to achieve maximal rates of translation of mRNA to inhibit/prevent protein folding that would be needed to achieve prM interaction with E and ZIKV assembly.

Figures 11A and 11B. Nucleotide and amino acid sequences for NS4A (Figure 11A). First line – original codon sequences in ZIKV; second line – codons optimized for good expression in *Salmonella*. Figure 11B is the codon-optimized sequence encoding a histidine tagged NS4A.

Figures 12A and 12B. Nucleotide and amino acid sequences for NS4B (Figure 12A). First line – original codon sequences in ZIKV; second line – codons optimized for good expression in *Salmonella*. Figure 12B is the codon-optimized sequence encoding a histidine tagged NS4B.

Figure 13. Plasmid map for pYA4589 containing NS4A.

Figure 14. Plasmid map for pYA4589 containing NS4B.

Figures 15A and 15B. Nucleotide and amino acid sequences for prM-E fusion protein (Figure 15A). First line – original codon sequences from ZIKV; second line – codons optimized for good expression in *Salmonella*. Figure 15B is the codon-optimized sequence encoding a histidine tagged prM-E fusion protein.

Figure 16. Plasmid map for pG8R126.

DETAILED DISCLOSURE OF THE INVENTION

One aspect of the present invention encompasses genetically modified recombinant facultative intracellular invasive bacterial pathogens (RFIIBPs), for example, a recombinant attenuated *Salmonella* vaccine (RASV) strain, capable of synthesizing ZIKV antigens. These genetically modified recombinant facultative intracellular invasive bacterial pathogens, when administered to a host, elicit an immune response against the ZIKV antigens. In particular embodiments, the genetically modified recombinant facultative intracellular invasive bacterial pathogens, for example RASV, colonize a host to substantially the same extent as a wild-type bacterium of the same serotype. The genetically modified recombinant facultative intracellular invasive bacterial pathogen is self-attenuating and becomes substantially avirulent after colonization. In certain embodiments, the recombinant facultative intracellular

invasive bacterial pathogen is a recombinant attenuated *Salmonella* vaccine (RASV) strain. The RASV strains may be any *Salmonella* bacterium, such as a *Salmonella enterica* serovar, a *S.* Typhimurium strain, *S.* Typhi strain, *S.* Paratyphi A strain, *S.* Gallinarum strain, *S.* Enteritidis strain, *S.* Choleraesius strain, or *S.* Dublin strain. In an exemplary embodiment, a bacterium of the invention may be from a *S.* Typhimurium or *S.* Typhi or *S.* Paratyphi A strain. Non-limiting examples of recombinant facultative intracellular invasive bacterial pathogens include: *Legionella pneumophila; Edwardsiella* spp*; Francisella tularensis*; *Yersinia* spp; *Mycobacterium tuberculosis; Listeria monocyotogenes; Salmonella* spp; invasive *Escherichia coli; Neisseria* spp*; Brucella* spp; or *Shigella* spp. In particular embodiments, recombinant facultative intracellular invasive bacterial pathogen or RASV comprising vaccines with plasmids encoding synthesis of non-glycosylated ZIKV capsid (C), membrane precursor (prM), and envelope (E) proteins together or individually are provided (and can be either native ZIKV sequences or codon optimized sequences such as those provided in Figures 3-6). In addition, recombinant facultative intracellular invasive bacterial pathogen or RASV comprising DNA vaccines encoding synthesis of non-glycosylated proteins can contain deletion of the glycosylation sites such that the open reading frame is not altered (e.g., 3, 6, 9, 12 or more nucleotides of the DNA vaccine are deleted) or the glycosylation motif (Asn-X-Thr or Asn-X-Ser where X is any amino acid) is altered such that the Asn residue is conservatively substituted with a Gln, His, Lys or Arg residue and/or the Thr or Ser residue can be substituted with a Val or Ala residue. In preferred embodiments, the Asn residue is conservatively substituted. The recombinant facultative intracellular invasive bacterial pathogen or RASV, preferably, undergo regulated delayed lysis in the *Salmonella* containing vesicle (SCV) (or a vesicle containing the recombinant facultative intracellular invasive bacterial pathogen) and cytosol and release synthesized ZIKV antigens to induce both cellular and antibody immune responses. This can result in antibodies to one or more of the non-glycosylated proteins that will not cross-react with any of the four DENV types.

These antigens can be encoded by codon-optimized or non-codon optimized polynucleotides for expression in the RFIIBPs or RASVs (with codon-optimized sequences encoding the antigens being preferred). Polynucleotides encoding antigens that are codon-optimized or non-codon optimized can also, optionally, be engineered to contain codons that cause translational delay as discussed below. The recombinant facultative intracellular invasive bacterial pathogen or RASV comprising DNA vaccines preferably, undergo regulated

delayed lysis after escape from the *Salmonella* containing vesicle (SCV) (or a vesicle containing the recombinant facultative intracellular invasive bacterial pathogen) to release the DNA vaccine encoding ZIKV antigens into the cytosol for migration to the host cell nucleus for expression of encoded ZIKV antigen genes to result in their synthesis by the immunized host. The use of codon-optimized sequences in the context of this aspect of the invention (in DNA vaccine constructs) is not necessary.

In certain embodiments, codons are optimized in the polynucleotides encoding the ZIKV antigens in a manner that enables higher levels of ZIKV antigen synthesis in the genetically modified bacterial pathogens, for example, *Salmonella*. In one embodiment, only certain codons are optimized for expression in the host bacterium while certain other codons that cause translational pauses are retained to permit protein folding of the antigens (Fluman et al., Komar et al., Kimchi-Sarfaty et al., Cortazzo et al., and Gingold et al.). The references Fluman et al., Komar et al., Kimchi-Sarfaty et al., Cortazzo et al., and Gingold et al. are incorporated by reference in their entirety.

In another embodiment, certain sequences that pause translation are introduced into the genes that encode ZIKV antigens, for example, the sequences described by Fluman et al. The sequences that pause translation can be located upstream or downstream of the codon being translated, particularly, 5-20 nucleotides upstream or downstream, preferably, 6-15 nucleotide upstream or downstream, and more preferably, 8-12 nucleotides upstream or downstream.

In a further embodiment, the sequences that pause translation are introduced into the genes that encode ZIKV antigens, for example, as described by Komar et al. Accordingly, some or all codons encoding a ZIKV antigen are replaced by rare codons that encode the same amino acids. A skilled artisan can determine rare codons in a particular organism and mutate a nucleotide sequence encoding a ZIKV antigen to produce a nucleotide sequence containing rare codons that encodes the same ZIKV antigen.

In an even further embodiment, the sequences that pause translation are introduced into the genes that encode ZIKV antigens, for example, as described by Gingold et al., particularly, as described in Figure 3 of Gingold et al. Accordingly, genes that encode ZIKV antigens are designed to contain specific upstream and downstream sequences relative to the start codon. For example, upstream sequences up to –15 nucleotides are designed to contain specific percentage of adenine nucleotides, particularly, between 30-50%, preferably, between 35-45%, and more preferably, about 40% adenine nucleotides. Also, downstream nucleotides at +4-6 positions are adenine or thymine. Further, downstream nucleotide at +15 can preferably be

thymine. The percentage of adenine nucleotides in the upstream sequence, downstream nucleotides at +4-6 positions and downstream nucleotide at +15 position can be modified as described herein in isolation or in any combination thereof.

Permitting proper folding induces antibody responses that neutralize ZIKV infection. However, certain antibodies cross reacting with DENV (based on conformational epitopes that are antigenically similar to DENV antigens) may be observed as long as the antigenicity of the conformational epitopes does not depend on post-translational modification such as glycosylation.

A further embodiment of the invention provides polynucleotides that encode ZIKV antigens that decrease or prevent the recombinant antigens synthesized by the genetically modified bacterial pathogens of the invention, for example, RFIIBPs or RASVs, from folding into secondary structures. As noted above, folding of ZIKV antigens can induce antibodies to conformational epitopes present in the ZIKV antigens folded into secondary structures. Therefore, preventing folding of ZIKV antigens reduce induction of cross reactive antibodies to conformational epitopes that may be similar in antigens from various flaviviruses such as ZIKV and DENV.

Accordingly, an embodiment of the invention provides polynucleotides that encode linear ZIKV antigens, *i.e.*, ZIKV antigens containing linear epitopes. The ZIKV antigens encoded by such polynucleotides do not fold into ZIKV antigens having secondary structures. Therefore, the linear ZIKV antigens of the invention induce high antibody titers that neutralize ZIKV to prevent infection; however, since the linear ZIKV antigens do not fold into antigens having secondary structures, the ZIKV linear antigens do not induce cross reacting antibodies that may be induced by conformational epitopes of the folded ZIKV antigens. Also, since the linear ZIKV antigens do not fold into antigens having secondary structures, the linear ZIKV antigens are secreted from the genetically modified bacterial pathogens without the need for chaperons. In one embodiment, two of the ZIKV surface antigens (prM and E) known to induce cross reactive antibodies were modified to produce polynucleotides that encode linear ZIKV antigens that do not exhibit protein folding.

In one embodiment, the polynucleotides that encode for ZIKV antigens containing linear epitopes, all the codons are optimized for expression in the host bacterium to allow robust expression of the antigens in the genetically modified bacterial pathogens, for example, *Salmonella.* Having all the codons optimized for expression prevents the pauses in translation due to scarcity of charged tRNA species and thus, accelerates translation. Accelerated

translation precludes folding of the antigens and enhances secretion of antigens fused to a type II secretion sequence, which causes induction of antibodies that do not recognize conformational epitopes on ZIKV and that neutralize ZIKV infection. The antibodies induced by the linear ZIKV antigens do not cross react with other flaviviruses, especially DENV because of the lack of conformational similarity between the linear ZIKV antigens described herein and DENV antigens having conformational epitopes.

In one embodiment, polynucleotides that encode for ZIKV antigens are provided, wherein the polynucleotides have all codons or a majority of codons changed to represent the most highly used codon (or two or more highly used codons) in the host bacterium for each amino acid. The "majority of codons" as used herein, refer to at least 80% to at least 99% of codons, particularly, at least 85% to 95% of codons, and more particularly, at least 90% of codons. These sequences encode mRNAs that are translated very rapidly into antigens that will not fold as they would during ZIKV maturation (see, for example, Figs. 10A and 1B). These antigens induce antibodies with high specificity to ZIKV and low reactivity with other flaviviruses. These antigens also exhibit enhanced secretion when combined with an improved β-lactamase signal sequence. In a particular embodiment, all codons or a majority of codons are changed to represent the most highly used codon (or codons) for highly expressed genes in *Salmonella*.

In other aspects, the subject application provides a recombinant facultative intracellular invasive bacterial pathogen or RASV comprising a DNA vaccine encoding a modified E sequence that induces neutralizing antibodies against ZIKV that should not cross-react with DENV. In some embodiments, the DNA vaccine contains an envelope (E) sequence wherein the 154 bp fragment that encodes the glycosylation motif, which is associated with virulence, has been deleted. Alternatively, the DNA vaccine can encode an E sequence that contains an altered glycosylation motif (Asn-X-Thr or Asn-X-Ser where X is any amino acid) such that glycosylation of the E sequence does not occur. For example, the Asn and/or Thr residue can be conservatively substituted with an amino acid as set forth in Table 1. For example, the Asn residue can be substituted with a Gln, His, Lys or Arg residue and/or the Thr or Ser residue can be substituted with an Ala or a Val residue. The Thr or Ser residue can, alternatively, be substituted with a Gly residue. In preferred embodiments, the Asn residue is conservatively substituted. Alternatively, the DNA vaccines contain deletion of the glycosylation sites such that the open reading frame is not altered (e.g., 3, 6, 9, 12 or more nucleotides of the DNA vaccine are deleted). The recombinant facultative intracellular

invasive bacterial pathogen or RASV, preferably, undergo regulated delayed lysis in the SCV and cytosol and induce both cellular and antibody immune responses will likely yield antibodies to one or more of the non-glycosylated proteins that will not cross-react with any of the four DENV types.

Yet other embodiments provide for recombinant facultative intracellular invasive bacterial pathogens or RASV that synthesize other ZIKV antigens. These ZIKV antigens can be synthesized, either individually or in combination, by the RASV disclosed herein and can be native or codon optimized sequences (such as those in Figures 3-6). These include: NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 or fusion proteins comprising two or more of these antigens. For example, a ZIKV polyfusion protein comprising NS1-NS2A-NS2B-NS3 or ZIKV NS4A-2K-NS4B-NS5 can be synthesized by the disclosed RASV or recombinant facultative intracellular invasive bacterial pathogen. Alternatively, individual antigens can be synthesized on individual plasmids or in a single plasmid under the control of a single promoter or separate promoters.

RFIIBPs and/or RASVs disclosed herein can be constructed using the teachings of this application and those of, for example, U.S. Patent Application Publication 2013/0171190A1 and U.S. Patent Application Publication 2012/0087946A1, each of which is hereby incorporated by reference in its entirety. Additional teachings can be found in the following references, each of which is also hereby incorporated by reference in its entirety: AMEISS, K. *et al.* "Delivery of woodchuck hepatitis virus-like particle presented influenza M2e by recombinant attenuated Salmonella displaying a delayed lysis phenotype" *Vaccine,* 2010, pp. 6704-6713, Vol. 28; ASHRAF, S. *et al.* "Protective cellular responses elicited by vaccination with influenza nucleoprotein delivered by a live recombinant attenuated Salmonella vaccine" *Vaccine,* 2011, pp. 3990-4002, Vol. 29; CURTISS, R. *et al.* "New Technologies in Using Recombinant Attenuated *Salmonella* Vaccine Vectors" *Critical Reviews™ in Immunology,* 2010, pp. 255-270, Vol. 30, No. 3; CURTISS, R. *et al.* "NONRECOMBINANT AND RECOMBINANT A VIRULENT *SALMONELLA* LIVE VACCINES FOR POULTRY" *Colonization Control of Human Bacterial Enteropathogens in Poultry,* 1991, pp. 169-198; JUÁREZ-RODRÍGUEZ, M. D. *et al.* "Live Attenuated *Salmonella* Vaccines against *Mycobacterium tuberculosis* with Antigen Delivery via the Type III Secretion System" *Infect. Immun.*, 2012, pp. 798-814, Vol. 80, No. 2; KONG, W. *et al.* "Regulated programmed lysis of recombinant Salmonella in host tissues to release protective antigens and confer biological containment" *Proceedings of the National Academy*

of Sciences of the United States of America, July 8, 2008, pp. 9361-9366, Vol. 105, No. 27; KONG, W. *et al.* "Turning self-destructing Salmonella into a universal DNA vaccine delivery platform" *Proceedings of the National Academy of Sciences of the United States of America,* October 11, 2012, pp. 1-13; KONG, W. *et al.* "Utilizing *Salmonella* for antigen delivery: the aims and benefits of bacterial delivered vaccination" *Expert Rev. Vaccines,* 2013, pp. 345-347, Vol. 12, No. 4; WANG, S. *et al.* "New technologies in developing recombinant attenuated Salmonella vaccine vectors" *Microbial Pathogenesis,* 2013, pp. 17-28, Vol. 58; and JIANG, Y. *et al.* "Protection Against Necrotic Enteritis in Broiler Chickens by Regulated Delayed Lysis *Salmonella* Vaccines" *Avian Diseases,* 2015, pp. 475-485, Vol. 59.

The RFIIBPs or RASVs disclosed herein contain one or more vectors, preferably one vector, that manipulates the ability of the RFIIBPs or RASVs to synthesize various essential constituents needed for synthesis of the rigid peptidoglycan layer of its cell wall (exemplary vector maps are provided in Figures 1 and, as one skilled in the art would recognize, the base vectors can be modified to contain a DNA sequence encoding an antigen of interest that is to be synthesized by the RFIIBPs or RASVs disclosed herein). The plasmids illustrated in Figure 1 have identical selective markers that permit a single vector to be stably maintained in the RASV at any given time. Where additional vectors are contemplated for use in the disclosed RFIIBPs or RASVs, one can introduce Δ*alr* and Δ*dadB* mutations and use vectors containing a $dadB^+$ gene. This produces a second balanced-lethal vector system based on the required need for D-alanine for peptidoglycan synthesis. It should also be noted that, some of the lysis vectors in Figure 1 (specifically Fig. 1A, 1C and 1D) are designed with a bacterial promoter P_{trc} to enable expression of inserted coding sequences and synthesis of the antigen by *Salmonella* to be delivered to the host. In contract, the vector in Fig. 1B is a DNA vaccine vector with a eukaryotic promoter P_{CMV} so that inserted genes are expressed in the eukaryotic host cells. The vectors in Figs. 1E, 1F and 1I are, therefore plasmids that cause synthesis of the antigens by the RASV or RFIIBPs whereas the vectors in Fig. 1G and IH specify antigens to be synthesized by the immunized host.

In one example, the constituent is diaminopimelic acid (DAP) and or muramic acid. Various enzymes are involved in the eventual synthesis of DAP. In one example, the RFIIBPs or RASVs are modified by using a Δ*asdA* mutation to eliminate the ability to produce β-aspartate semialdehyde dehydrogenase, an enzyme essential for the synthesis of DAP. One of skill in the art can also use the teachings of U.S. Pat. No. 6,872,547 for other

types of mutations of nucleic acid sequences that result in the abolition of the synthesis of DAP. These nucleic acid sequences may include, but are not limited to, *dapA, dapB, dapC, dapD, dapE, dapF*, and *asdA*. The disclosed RFIIBPs or RASVs may comprise (or further comprise) a genetic modification such that the synthesis of another essential constituent of the rigid layer of the bacterial cell wall is dependent on a nutrient (e.g., arabinose) that can be supplied during the growth of the microorganism. However, when arabinose is absent as it is in an animal or human host, the essential constituents of the peptidoglycan layer of the cell wall are not synthesized. These mutations represent an arabinose dependent lethal mutation. In the absence of arabinose, synthesis of muramic acid and DAP ceases and lysis of the RFIIBPs or RASVs occurs because the peptidoglycan layer of the cell wall is not synthesized. RFIIBPs or RASVs with a ΔP_{murA}::TT *araC* P_{BAD} *murA* deletion-insertion mutation grown in the presence of arabinose exhibit effective colonization of effector lymphoid tissues after oral vaccination prior to undergoing lysis due to the inability to synthesize muramic acid. Similarly, various embodiments may comprise the *araC* P_{BAD} *c2* cassette inserted into the *asdA* nucleic acid sequence that encodes aspartate semialdehyde dehydrogenase in RFIIBPs or RASVs or deletion/inactivation of the chromosomal *asdA* gene encoding aspartate semialdehyde dehydrogenase to enable use of plasmid vectors encoding the wild-type *asdA* nucleic acid sequence.

In some embodiments, the promoter may be responsive to the level of arabinose in the environment, as described above. In other embodiments, the promoter may be responsive to the level of maltose, mannose, rhamnose, or xylose in the environment, as described above. The promoters detailed herein are known in the art, and methods of operably linking them to a nucleic acid sequence encoding an attenuation protein are known in the art. Generally speaking, the concentration of arabinose, maltose, mannose, rhamnose, or xylose necessary to induce expression is typically less than about 2%. In some embodiments, the concentration is less than about 1.5%, 1%, 0.5%, 0.2%, 0.1%, or 0.05%. In certain embodiments, the concentration may be about 0.04%, 0.03%, 0.02%, or 0.01%. In an exemplary embodiment, the concentration is about 0.05%.

RFIIBPs or RASVs may, optionally, comprise further genetic modifications. For example, the gene encoding phosphomannose isomerase may be deleted or inactivated in RFIIBPs or RASVs. Alternatively, the gene encoding O-antigen ligase (*waaL)* can be deleted or inactivated. Similarly, additional genetic alterations, such as inactivation or deletion of one or more of the following genes can be performed on the disclosed RFIIBPs or

RASVs: *relA*, *wza-wcaM*, *sifA*, *recF*, *recJ*, *sseL*, *tlpA*, *msbB* and *pagP*. RFIIBPs or RASVs disclosed herein can also be genetically modified to synthesize the non-toxic adjuvant form of LPS, lipid A monophosphoryl lipid A (MPLA) by introduction of a *lpxE* gene that is operably linked to a repressor sensitive promoter which facilitates delayed *in vivo* synthesis of MPLA by the RFIIBPs or RASVs. RFIIBPs or RASVs are capable of the regulated expression of at least one nucleic acid sequence and comprise a vector. The vector comprises a nucleic acid sequence encoding at least one antigen of interest operably linked to a promoter. The promoter is regulated by a repressor, such that the expression of the nucleic acid sequence encoding a least one flavivirus antigen (e.g., a ZIKV antigen) is repressed during *in vitro* growth of the RFIIBPs or RASVs, but the RFIIBPs or RASVs are capable of high level synthesis of the antigen in an animal or human host. Other genetic modifications include genetic modification of Shine Dalgarno sequences and/or the introduction of GTG start codons such that translational efficiency of mRNA synthesized by the RFIIBPs or RASVs is decreased (see, for example, U.S. Patent Application Publication 2013/0171190A1 and U.S. Patent Application Publication 2012/0087946A1 each of which is hereby incorporated by reference in this regard).

As used herein, "vector" refers to an autonomously replicating nucleic acid unit. The most preferred type of vector is a plasmid vector with examples illustrated in Figure 1. As is well known in the art, plasmids and other vectors may possess a wide array of promoters, multiple cloning sequences, transcription terminators, etc., and vectors may be selected so as to control the level of expression of the nucleic acid sequence encoding an antigen by controlling the relative copy number of the vector.

In some instances in which the flavivirus antigen is capable of stimulating T-cell immunity, it may be preferable to use a vector with a low copy number such as at least two, three, four, five, six, seven, eight, nine, or ten copies per cell. A non-limiting example of a low copy number vector may be a vector comprising the pSC101 *ori*. In other cases, an intermediate copy number vector might be optimal for inducing a desired immune response. For instance, an intermediate copy number vector may have at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 copies per cell. A non-limiting example of an intermediate copy number vector may be a vector comprising the p15A *ori*. In still other cases, a high copy number vector might be optimal for the induction of maximal antibody response to a flavivirus antigen (e.g., a ZIKV antigen). A high copy number vector may have at least 31, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 copies per cell.

In some embodiments, a high copy number vector may have at least 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, or 400 copies per cell. Non-limiting examples of high copy number vectors may include a vector comprising the pBR *ori* or the pUC *ori*. Additionally, vector copy number may be increased by selecting for mutations that increase plasmid copy number. These mutations may occur in the bacterial chromosome but are more likely to occur in the plasmid vector. Preferably, vectors used herein do not comprise antibiotic resistance markers to select for maintenance of the vector.

The vector may comprise a nucleic acid sequence encoding at least one flavivirus antigen (preferably a ZIKV antigen) operably-linked to a promoter regulated by the repressor. One of skill in the art would recognize, therefore, that the selection of a repressor dictates, in part, the selection of the promoter operably-linked to a nucleic acid sequence encoding an antigen of interest. For instance, if the repressor is LacI, then the promoter may be selected from the group consisting of LacI responsive promoters, such as P_{trc}, P_{lac}, P_{T7lac} and P_{tac}. If the repressor is C2, then the promoter may be selected from the group consisting of C2 responsive promoters, such as P22 promoters P_L and P_R. If the repressor is C1, then the promoter may be selected from the group consisting of C1 responsive promoters, such as λ promoters P_L and P_R.

In each embodiment herein, the promoter regulates expression of a nucleic acid sequence encoding the flavivirus (e.g., ZIKV) antigen, such that expression of the nucleic acid sequence encoding an antigen is repressed when the repressor is synthesized (i.e. during in vitro growth of the RFIIBPs or RASVs), but expression of the nucleic acid sequence encoding a flavivirus (e.g., ZIKV) antigen is high when the repressor is not synthesized (i.e. in an animal or human host). Generally speaking, the concentration of the repressor will decrease with every cell division after expression of the nucleic acid sequence encoding the repressor ceases. In some embodiments, the concentration of the repressor decreases enough to allow high level expression of the nucleic acid sequence encoding a flavivirus (e.g., ZIKV) antigen after about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 divisions of the RFIIBPs or RASVs. In an exemplary embodiment, the concentration of the repressor decreases enough to allow high level expression of the nucleic acid sequence encoding an antigen after about 5 divisions of the RFIIBPs or RASVs in an animal or human host.

In certain embodiments, the promoter may comprise other regulatory elements. For instance, the promoter may comprise lacO if the repressor is LacI. This is the case with the

lipoprotein promoter P_{lpp} that is regulated by LacI since it possesses the LacI binding domain *lacO*. In one embodiment, the repressor is a LacI repressor and the promoter is P_{trc}.

As detailed above, generally speaking the expression of the nucleic acid sequence encoding the antigen should be repressed when the repressor is synthesized. For instance, if the repressor is synthesized during in vitro growth of the RFIIBPs or RASVs, expression of the nucleic acid sequence encoding the antigen should be repressed. Expression may be "repressed" or "partially repressed" when it is about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or even less than 1% of the expression under non-repressed conditions. Thus although the level of expression under conditions of "complete repression" might be exceeding low, it is likely to be detectable using very sensitive methods since repression can never by absolute. Conversely, the expression of the nucleic acid sequence encoding the antigen should be high when the expression of the nucleic acid sequence encoding the repressor is repressed. For instance, if the nucleic acid sequence encoding the repressor is not expressed during growth of the RFIIBPs or RASVs in the host, the expression of the nucleic acid sequence encoding the antigen should be high. As used herein, "high level" expression refers to expression that is strong enough to elicit an immune response to the flavivirus (e.g., ZIKV) antigen. Methods of determining whether an antigen elicits an immune response such as by measuring antibody levels or antigen-dependent T cell populations or antigen-dependent cytokine levels are known in the art, and methods of measuring levels of expression of flavivirus (e.g., ZIKV) antigen encoding sequences by measuring levels of mRNA transcribed or by quantitating the level of antigen synthesis are also known in the art.

As discussed above, the disclosed recombinant facultative intracellular invasive bacterial pathogen or RASV can deliver synthesized ZIKV antigens specified by plasmids such as derivatives of pYA4763, pYA4589 and pYA4595 depicted in Figure 1 and introduced into *S.* Typhimurium strain χ11829 (ΔP_{murA25}::TT *araC* P_{BAD} *murA* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ*pmi-2426* Δ(*wza-wcaM*)-*8* Δ*relA197*::*araC* P_{BAD} *lacI* TT Δ*recF126* Δ*sifA26*) that possesses the regulated delayed antigen synthesis attribute and the regulated delayed lysis attribute. This strain also possesses the Δ*sifA* mutation so that lysis occurs in the cytosol to release synthesized ZIKV antigens for presentation to the proteasome for ultimate class I presentation to enhance induction of CD8- and CD17-dependent cellular immunities.

As discussed above, the disclosed recombinant facultative intracellular invasive bacterial pathogen or RASV can deliver DNA vaccines using vectors, such as derivatives of pYA4545 which are illustrated in Figure 1. Methods for constructing such DNA vaccines (as well as plasmids for delivering DNA vaccines) are described in Kong et al., 2012, *Proc. Nat'l. Acad. Sci. USA*, 109(47):19414-19419 (the disclosure of which is hereby incorporated by reference in its entirety). For example, specific embodiments provide RASV containing the following genetic modifications that can be used to deliver the DNA vaccines: ((ΔP_{murA}::TT *araC* P_{BAD} *murA;* Δ*asdA*::TT *araC* P_{BAD} *c2;* Δ(*wza-wcaM*) Δ*pmi* Δ*relA* Δ*recF* Δ*endA* Δ*sseL* Δ*tlpA* ΔP_{hilA}::$P_{trc\Delta lacO}$ *hilA*) and ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*pmi* Δ*waaL* Δ*pagL*::TT *rhaRS* P_{rhaBAD} *waaL* Δ(*wza-wcaM*) Δ*relA* Δ*endA* Δ*sseL* Δ*tlpA* Δ*recF* Δ*sifA*. In some embodiments, the RASV are *S.* Typhimurium strains χ11848 ((ΔP_{murA}::TT *araC* P_{BAD} *murA;* Δ*asdA*::TT *araC* P_{BAD} *c2;* Δ(*wza-wcaM*) Δ*pmi* Δ*relA* Δ*recF* Δ*endA* Δ*sseL* Δ*tlpA* ΔP_{hilA}::$P_{trc\ \Delta lacO}$*hilA*) and χ12386 ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*pmi* Δ*waaL* Δ*pagL*::TT *rhaRS* P_{rhaBAD} *waaL* Δ(*wza-wcaM*) Δ*relA* Δ*endA* Δ*sseL* Δ*tlpA* Δ*recF* Δ*sifA*.

In certain additional aspects of the application, a vaccine comprising the disclosed recombinant facultative intracellular invasive bacterial pathogens or RASV are provided. The disclosed recombinant facultative intracellular invasive bacterial pathogens or RASV can be administered to a host as a vaccine composition that is designed to induce an immune response to the antigens being delivered by the RASV. As discussed above, the subject application envisions induction of an immune response that is protective and generation of antibodies that react with ZIKV antigens but which do not substantially cross react with DENV antigens. As used herein, "protective" means that the immune response contributes to the lessening of any symptoms associated with infection of a host with ZIKV and need not imply that the immunized host is completely protected from the effects of the ZIKV. In certain embodiments, the vaccine is orally administered and induces protective mucosal immunity in the host (e.g., the mucosal immunity decreases the infectivity of ZIKV in bodily secretions). Vaccine compositions of the present invention may be administered to any host capable of mounting an immune response. Such hosts may include domesticated animals, agricultural animals, laboratory animals, and humans. A vaccine composition comprising a recombinant facultative intracellular invasive bacterial pathogen or RASV may, optionally, contain one or more additional components, such as carriers, preservatives, stabilizers,

adjuvants, and other substances. In another embodiment, the vaccine composition may also contain a pharmaceutical carrier (or excipient). Carriers and excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington's Pharmaceutical Sciences 19th Ed. Mack Publishing (1995).

Various embodiments contemplate the oral administration of the disclosed vaccine compositions. Typical dosages for oral administration can range from about 1×10^{7} to 1×10^{10} CFU. Administering multiple dosages may also be used as needed to provide the desired level of protective immunity. In order to stimulate an immune response, the disclosed recombinant facultative intracellular invasive bacterial pathogens or RASV compositions can be administered on mucosal surfaces, such as orally or intranasally (as a liquid or aerosol). Where the disclosed RASV are administered to humans, oral administration is preferred.

A further aspect of the invention encompasses methods of using the disclosed recombinant facultative intracellular invasive bacterial pathogens or RASV disclosed herein for inducing an immune response specific to ZIKV in a host. The method comprises administering an effective amount of a composition comprising a recombinant facultative intracellular invasive bacterial pathogen or a RASV disclosed herein to a host. An effective amount of a composition comprising a recombinant facultative intracellular invasive bacterial pathogen or a RASV as disclosed herein is an amount that will generate the desired immune response (e.g., mucosal, humoral and/or cellular) to ZIKV antigens synthesized by the RASV or the recombinant facultative intracellular invasive bacterial pathogen. Methods of monitoring a host's immune response are well-known to those skilled in the art and non-limiting examples include ELISA and ELISPOT assays. In particular embodiments, the antibodies induced by the disclosed RASV are not substantially cross reactive with, or does not substantially bind to, similar antigens from other flaviviruses, for example DENV.

The phrase "neutralizing antibodies against ZIKV that do not cross-react with DENV" is intended to convey that the antibodies neutralize the ability of ZIKV to initiate and/or cause an infection in a host and/or in target cells *in vitro*; however, these antibodies have no significant effect on the ability of DENV to initiate or cause an infection in a host and/or in target cells *in vitro*. Likewise, the phrases "not substantially cross reactive with", or "does not substantially bind to" similar antigens from other flaviviruses (such as any of the four DENV types) is intended to convey that the antibodies have the ability to neutralize the ability of ZIKV to initiate and/or cause an infection in a host and/or in target cells *in vitro*; however, these antibodies have no significant effect on the ability of any of the four DENV

types to initiate or cause an infection in a host and/or in target cells *in vitro*. For example, standard ZIKV or DENV plaque-reduction-neutralization tests can be used to determine if neutralizing antibodies to ZIKV antigens neutralize DENV infectivity in a host cell or a target cell.

The phrase "ZIKV protein antigens that may, optionally, be encoded by codon optimized nucleotide sequences and/or engineered for translational acceleration or delay" is meant to include the use of non-codon optimized nucleotide sequences encoding ZIKV protein antigens, the use of codon optimized nucleotide sequences encoding ZIKV antigens, the use of nucleotide sequences that are engineered for translational delay, the use of nucleotide sequences that are engineered for translational acceleration, or any combination of such nucleotide sequences that encode ZIKV protein antigens.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Materials and Methods

Vaccine vector strains. *S.* Typhimurium strains will be used to synthesize and deliver non-structural and non-glycosylated surface proteins to immunized animal hosts. χ11829 (ΔP_{murA}::TT *araC* P_{BAD} *murA;* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*pmi;* Δ(*wza-wcaM*) Δ*relA*::*araC* P_{BAD} *lacI* TT; Δ*recF* Δ*sifA*) and χ12341 ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*pmi* Δ*waaL* Δ*pagL*::TT *rhaRS* P_{rhaBAD} *waaL* Δ(*wza-wcaM*) Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*recF* Δ*sifA*. Two other strains will be used for delivery of DNA vaccine vectors encoding ZIKV surface proteins encoded by sequences modified to eliminate post-translational glycosylation of surface proteins synthesized within the immunized animal host. χ11848 ((ΔP_{murA}::TT *araC* P_{BAD} *murA;* Δ*asdA*::TT *araC* P_{BAD} *c2;* Δ(*wza-wcaM*) Δ*pmi* Δ*relA* Δ*recF* Δ*endA* Δ*sseL* Δ*tlpA* ΔP_{hilA}::$P_{trc\ \Delta lacO}$*hilA*) and χ12386 ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*pmi* Δ*waaL* Δ*pagL*::TT *rhaRS* P_{rhaBAD} *waaL* Δ(*wza-wcaM*) Δ*relA* Δ*endA* Δ*sseL* Δ*tlpA* Δ*recF* Δ*sifA* have been constructed. Both of these RASV have a regulatable *in vivo* delayed lysis phenotype and escape the SCV

to lyse in the cytosol to deliver antigens to the proteosome for MHC class I presentation or DNA vaccine vectors encoding antigens, respectively. The delivery of synthesized antigens to the proteosome induces CD8-, CD17- and NKT-dependent immune responses. The RASV strain χ11848 can be used to deliver DNA vaccines encoding protective antigens to the nucleus of host cells for transcription. *S.* Typhi strains of the same genotypes for use in human vaccines will be generated. These strains will include a Δ*pagP*::P_{trc} *lpxE* mutation specifying synthesis of non-toxic adjuvant monophosphoryl lipid A. See KONG, Q. *et al,* "*Salmonella* Synthesizing 1-Monophosphorylated Lipopolysaccharide Exhibits Low Endotoxic Activity while Retaining Its Immunogenicity" *The Journal of Immunology*, 2011, pp. 412-423, Vol. 187.

Plasmid vectors. All plasmids to be used confer the regulated delayed lysis *in vivo* phenotype and employ the balanced-lethal vector-host concept to ensure that live RASVs would be sensitive to all antibiotics and thus unable to disseminate antibiotic resistance when RASVs were used in open agricultural settings. The regulated lysis vectors depicted in Figs. 1A, 1C, 1D-1F and 1I all have P_{trc} regulated synthesis of protective antigens for delivery by cell lysis and *araC* P_{BAD} regulated *murA* and *asd* genes with GTG start codons to decrease translation efficiency. Certain of these vectors also contain modified Shine Dalgarno sequences that also affect translational efficiency (e.g., Figs. 1C). The P22 P_R located with opposite orientation to the transcription of the *araC* P_{BAD} GTG-*murA* GTG-*asd* genes is repressed by the C2 repressor made during growth of the strain with arabinose. However, C2 concentration decreases due to cell division *in vivo* to cause P_R-directed anti-sense mRNA synthesis to block translation of residual *asdA* and *murA* mRNA. Transcription terminators (TT) flank all plasmid domains for controlled lysis, replication and gene expression so that expression in one domain does not affect activities of another domain.

Bacterial strains, media and bacterial growth. All RASVs are derived from the highly virulent *S.* Typhimurium strain UK-1 (Curtiss *et al.*, 1991) that are more immunogenic and superior to vaccines derived from other *S.* Typhimurium strains (Zhang *et al.*, 1997). LB broth and agar (Bertani, 1951) will be used as complex media for propagation and plating of *Salmonella* strains. Purple broth (Difco) and MacConkey agar with 0.5% lactose will be used as necessary.

Molecular and genetic procedures. Methods for DNA isolation, restriction enzyme digestion, DNA cloning, and use of PCR for construction and verification of vectors and strains are standard (Sambrook, 2001). All DNA syntheses will be done commercially with

codon optimization to enhance translational efficiency in *Salmonella* and to stabilize mRNA by destroying (removing) RNase E cleavage sites thereby prolonging mRNA half-life (Ehretsmann *et al.*, 1992; McDowall *et al.*, 1995; Lin-Chao *et al.*, 1994). Plasmids will be sequenced and tested for ability to specify synthesis of proteins using gel electrophoresis and western blot analyses. DNA vaccine constructs will be similarly tested after electroporation into Vero cells.

Strain characterization. *S.* Typhimurium strains χ11829,and χ12341 will be electroporated with the constructed antigen-encoding lysis vectors (Figure 1E (base vector of Fig. 1D containing nucleic acid sequence encoding codon optimized envelope (E) protein) and 1F (base vector of Fig. 1D containing nucleic acid sequence encoding codon optimized envelope (E) protein and amino acid substitutions at ASN154 and ASN481) and 1I (base vector containing nucleic acid sequence encoding a fusion protein of membrane precursor (prM) and NS5 (each of which is not codon optimized for χ11829 and χ12341) and χ11848 with DNA vaccine vectors (illustrated in Figures 1B (base vector), 1G (base vector containing nucleic acid sequence encoding non-codon optimized envelope (E) protein) and 1H (base vector containing nucleic acid sequence encoding codon optimized envelope (E) protein and amino acid substitutions at ASN154 and ASN481 for χ11848), respectively, and evaluated in comparison with strains containing empty vector controls (e.g., vectors illustrated in Figures 1C (base vector (empty control vector) with modified Shine Dalgarno sequences), 1D (base vector; empty control vector) for χ11829 and 1B (base vector; empty control vector) for χ11848). Metrics will include: stability of plasmid maintenance, integrity, and antigen synthesis ability when strains are grown in the presence of arabinose and DAP for 50 generations (Galán *et al.*, 1990). Final RASVs will be evaluated for synthesis of LPS O-antigen (Hitchcock *et al.*, 1983), bile sensitivity, acid tolerance, and ability to survive in sera with and without complement inactivation and in macrophages (Curtiss *et al.*, 2009; Shi *et al.*, 2009). These tests will be conducted with RASVs grown with and without 0.1 % media supplementation with mannose. Sensitivity to all antibiotics used to treat *Salmonella* infections will also be validated. Strain metabolic attributes will be evaluated using API-20E tests. DNA vaccine constructs will be transfected into Vero cells to evaluate synthesis and masses of encoded protective antigens in the presence and absence of tunicamycin to block glycosylation.

Viruses. ZIKV MR-766 (original-African), ZIKV PRVABC59 (outbreak-Asian),

DENV-1-HI, DENV-2 NGC, DENV-3 H87, and DENV-4 H241tc will be used. ZIKV PRVABC59 was used to design plasmid constructs.

Antigen preparation: Viral antigens specified by RASVs will be synthesized as His-tagged proteins in recombinant *E. coli*. The *E. coli* used lacks flagella, fimbrial antigens, and LPS O-antigen to enhance purity of recombinant proteins. Glycosylated viral proteins will be isolated from ZIKV, synthesized, or obtained commercially. *Salmonella* B group LPS O-antigens will be obtained commercially. *S.* Typhimurium outer membrane protein (SOMP) fraction and heat-killed extracts of the wild-type parental *Salmonella* strain used to construct RASVs will be used as controls in western blots and for immunoassays.

ELISA: Serum antibodies from mice will be measured in blood collected from mice and a doubling dilution method, with the end-point titer being the dilution giving an OD_{450} three times that for the reagent or unimmunized animal control, will be employed. Titers of IgG1 and IgG2a will be determined to distinguish between Th1 and Th2 responses. ZIKV antigens will be obtained from recombinant proteins and whole virus. Mucosal antibody secretion will be measured by ELISPOT analyses and in vaginal secretions by ELISA.

Flow cytometry analysis: Flow cytometry will be used to quantitate populations of CD4+, CD8+ and CD17+ T cells and specific cytokine-secreting cells in peripheral blood, kidneys, and spleens of mice immunized with RASV strains delivering protective antigens or DNA vaccines encoding viral antigens. Single cell suspensions from kidney and spleens of individual mice will be suspended in PBS in the presence of purified antigens and incubated with specific antibodies for detection of cell surface molecules. Presence of CD4, CD8, CD11a, CD17, CD44, and CD62L markers on cell surfaces will be assessed using fluorescence-labeled antibodies specific for each marker. Intracellular IFN-γ, TNF-α, IL-17, and IL-10 production by CD4+ T cells will also be measured using specific antibodies. All samples will be analyzed on a Becton Dickinson LSR Fortessa instrument and data analyzed using FCS Express 4 Flow Research Edition software.

T-cell Proliferation Assays/CTL responses: Will be performed as described in (Kappes *et al.*, 2012).

Virus neutralization assays: To determine if the RASV constructs induce neutralizing antibody responses, ZIKV-neutralizing properties of sera from vaccinated and non-vaccinated mice will be tested. Sera will be assayed using standard ZIKV or DENV plaque-reduction-neutralization tests.

Enhancement and Antibody Dependent Enhancement-Inhibition Assays: Whole serum from RASV-immunized and non-immunized mice will be studied to determine how antibodies to ZIKV behave upon encounter with DENV. These assays will be performed as described in the literature using human macrophage/PBMC cells (Nicholson *et al.*, 2011; Sasaki *et al.*, 2013). The results will be analyzed using the ΔΔ Ct method.

Statistical analyses. All results will be analyzed using the most appropriate statistical tests from the SAS program to evaluate the relative significance or lack thereof of results obtained.

EXAMPLE 1— Procedural overview for constructions and evaluations

RASVs delivering DNA vaccine vectors and RASVs synthesizing protein antigen from antigen-encoding lysis vectors will be produced as described above in the "**Strain characterization"** section. Five RASVs delivering ZIKV DNA vaccines, a vector control and a buffered saline control will be administered to groups of female BALB/c mice (10 animals per group). The immunization protocol comprises orally administering 10^9 CFU on days 0, 7, and 49 and a repeat experiment of a longer duration that uses the same or a lower dose will be also be performed before giving the booster immunization. Sera will be collected on days -1, 14, 28, 42, 56, and 70. Mucosal and serum antibody titers to each of the three ZIKV surface antigens and to *Salmonella* LPS and OMPs will be determined using ELISA. Neutralizing antibody titers against ZIKV and DENV types 1-4 will be determined via virus neutralization assays.

At 70 days, mice will be euthanized and peripheral blood and spleen cells evaluated for antigen-specific lymphocyte proliferation, cytokine production, and CD4-, CD8- and NKT-dependent responses using tetramers. ELISA and/or plaque reduction neutralization test (PRNT) assays will be used to identify any cross-reactivity or enhancing effects of immune responses with DENV types 1-4. PRNT will be used, as well as enhancement and antibody dependent enhancement-inhibition assays, to identify and describe any virus-neutralizing and/or enhancing properties.

RASVs containing antigen-encoding lysis vectors that direct synthesis of non-glycosylated C, prM and E proteins will also be evaluated. These antigen-encoding lysis vectors will contain codon-optimized sequences encoding the three ZIKV surface proteins individually and one encoding all three proteins. Each of these four plasmids will be electroporated into χ11829 or χ12341. The four RASVs synthesizing and delivering ZIKV

surface antigens will be evaluated in groups containing10 mice per experimental and control group. We will orally immunize BALB/c mice on days 0, 7, and 49 with each recombinant vaccine and collect sera on days -1, 14, 28, 42, 56, and 70. ELISA will be used to determine antibody titers to each of the three surface antigens and to *Salmonella* LPS and OMPs. We will also determine whether any antibodies neutralize ZIKV but fail to react with any DENV surface proteins via ELISA and virus neutralization assays. At 70 days, we will euthanize the mice and evaluate peripheral blood and spleen cells for antigen-specific lymphocyte proliferation, cytokine production and CD4-, CD8- and NKT-dependent responses using tetramers. Additional experiments will be conducted to assess the presence of specificity for ZIKV proteins and the absence of cross-reactivity or enhancing effects of immune responses with proteins from DENV types 1-4 using ELISA and virus neutralization assays as well as enhancement and antibody dependent enhancement-inhibition assays. We will also examine whether ZIKV-specific T-cells will kill ZIKV-infected Vero cells.

Additional experiments using RASVs containing antigen-encoding lysis vectors that direct synthesis of non-glycosylated ZIKV NS1-NS2A-NS2B-NS3 as a polyprotein fusion and ZIKV NS4A-2K-NS4B-NS5 as a polyprotein fusion or individual NS1, NS2A, NS2B, NS3, NS4A, NS4b and NS5 will also be evaluated. These antigen-encoding lysis vectors will contain codon-optimized sequences encoding the individual or polyproteins. Each of these plasmids will be electroporated into χ11829 or χ12341. The RASVs synthesizing and delivering ZIKV NS proteins will be evaluated in groups containing 10 mice per experimental and control groups (immunized with an empty (base) lysis vector and buffer). We will orally immunize BALB/c mice on days 0, 7, and 49 with each recombinant vaccine and collect sera on days -1, 14, 28, 42, 56, and 70. ELISA will be used to determine antibody titers to each of the seven non-structural proteins and to *Salmonella* LPS and OMPs will be quantified via ELISA. We will determine whether any antibodies neutralize ZIKV and fail to react with any DENV surface proteins via ELISA and/or virus neutralization tests. At 70 days, we will euthanize the mice and evaluate peripheral blood and spleen cells for antigen-specific lymphocyte proliferation, cytokine production and CD4-, CD8- and NKT-dependent responses using tetramers of the non-structural proteins. We will also examine whether ZIKV-specific T-cells to the non-structural proteins will kill ZIKV-infected Vero cells.

EXAMPLE 2 – Construction of lysis vectors encoding NS proteins

We inserted the codon optimized sequences for NS4A and NS4B (lower sequences in Figures 11 and 12) into the pYA4589 (p15A *ori*) vector (Figure 1D) to yield pG8R124 (Figure 13) and pG8R125 (Figure 14), respectively. However, initially both sequences with a C-terminal 6-His tag, to enable measurement of levels of protein synthesis using the mouse monoclonal poly-histidine antibody clone HIS-1 (Sigma H1029), were inserted into pYA4589 and the sequences encoding the 6-His residues deleted by plasmid digestion with NaeI (see sequences at the bottom of Figures 11 and 12). All plasmids were initially electroporated into *E. coli* K-12 χ6212(pYA232) (F^- λ^- φ80 Δ(*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4* with pYA232 encoding $lacI^q$) with selection for Asd^+ in the presence of 0.1% arabinose. Asd+ isolates were tested for presence of the plasmid of the correct size and content and ability to grow in the presence of IPTG to relieve the LacI repression of transcription from the plasmid P_{trc} to ensure that synthesis of the encoded ZIKV protein was not toxic. The recombinant plasmids were then electroporated into the *S.* Typhimurium χ12341 and growth evaluated in the presence and absence of IPTG. Recombinant clones with the His-tagged sequence were evaluated for synthesis if the His-tagged ZIKV proteins. All RASV strains were tested for synthesis of LPS O-antigen dependent on presence of mannose and rhamnose. This verified that rough variants were not selected during the electroporation operation. RASVs were then tested for stability of plasmid maintenance and ability to synthesize the ZIKV antigen after 50 generations of growth under permissive conditions in DAP-supplemented LB broth. χ12341(pG8R124) and χ12341(pG8R125) were then judged to satisfy all criteria to advance toward evaluation in mice.

EXAMPLE 3 – Construction of lysis vectors encoding prM-E fusion protein

The immune response to the prM-E fusion should result from a combined production of neutralizing antibodies and cellular immunity. Since some cross reactive antibodies might be due to glycosylation, the synthesis and delivery of this fusion protein from *Salmonella* should eliminate such glycosylation even without alteration of the N-X-S/T glycosylation sites in the E encoding sequence. As in Example 2, the codon-optimized sequence for the prM-E fusion (Figure 15) with the C-terminal 6-His tag was inserted into pYA4589, which was then electroporated into χ6212(pYA232). The NaeI digested plasmid without the His tag

was also electroporated into χ6212(pYA232) and designated pG8R126 (Figure 16). All of the procedures for plasmid characterization were as described in Example 2 both before and after introducing pG8R126 into χ12341. χ12341(pG8R126) is thus ready for studies in mice as described in the Material and Methods section.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated within the scope of the invention without limitation thereto.

Table 1. Mutations and associated phenotypes in *S.* Typhimurium vaccine strains[a]

Genotype	Phenotype
A. Deletion and deletion-insertion mutations to confer regulated delayed in vivo attenuation phenotype	
Δ*pmi*	encodes phosphomannose isomerase needed for synthesis of GDP-mannose for LPS O-antigen and thus necessary for virulence
Δ*waaL* Δ*pagL*::TT *rhaRS* P_{rhaBAD} *waaL*	regulates synthesis of enzyme responsible for attaching first subunit of LPS O-antigen to the LPS core (the deletion of the *waaL* gene is necessary to prevent impairment in expression of other *rfb* operon genes); the regulated expression cassette is therefore inserted into the *pagL* gene.
B. Promoters and deletion-insertion mutations for regulated delayed in vivo synthesis of antigens	
P_{trc}	a promoter expressed at high level under both anaerobic and aerobic conditions and repressed by LacI
Δ*relA*::*araC* P_{BAD} *lacI* TT	The arabinose-dependent synthesis of the LacI repressor is to enable a regulated delayed expression of DNA sequences under the control of P_{trc}
Phage P22 P_R	promoter is repressible by arabinose-dependent synthesis of the C2 repressor
C. Deletion and deletion-insertion mutations to facilitate regulated delayed lysis in vivo	
ΔP_{murA}::TT *araC* P_{BAD} *murA*	makes synthesis of MurA, the first enzyme in the synthesis of muramic acid, dependent on arabinose in growth medium and ceases synthesis in vivo due to absence of arabinose. MurA decreases due to cell division in vivo to ultimately lyse and die. The *murA* defect is complemented by $MurA^+$ plasmid vectors.
Δ*asdA*::TT *araC* P_{BAD} *c2*	the Asd enzyme is essential for the synthesis of diaminopimelic acid (DAP) required for peptidoglycan synthesis. The arabinose-dependent synthesis of the C2 repressor enables a regulated delayed expression of DNA sequences under control of C2 repressed promoters. The Δ*asdA* mutation is complemented by Asd^+ plasmids
Δ*relA*	the *relA* mutation uncouples growth regulation from a dependence on protein synthesis, an important attribute in strains with regulated delayed lysis
Δ(*wza-wcaM*)	eliminates twenty enzymes needed to synthesize several exopolysaccharides that promote biofilm formation and synthesis of GDP-fucose required for colanic acid synthesis, which protects cells undergoing cell wall-less death from lysing
D. Other contributing mutations	
Δ*sifA*	enables *Salmonella* to escape the SCV for lysis in cytosol

Δ*recF* & Δ*recJ*	eliminate recombinases facilitating inter- and intra-plasmidic recombination
Δ*sseL* Δ*tlpA*	decrease and delay *Salmonella*-induced pyroptosis/apoptosis
Δ*msbB*	alters LPS lipid A to render it less toxic
Δ*pagP*::P_{trc} *lpxE* mutation	causes regulated delayed in vivo synthesis of the codon-optimized *lpxE* gene from *Francisella tularensis* to cause synthesis of the non-toxic adjuvant form of LPS lipid A monophosphoryl lipid A (MPLA)

[a] Δ = deletion; TT = transcription terminator; P = promoter

REFERENCES

Curtiss R, III., S. B. Porter, M. Munson, S. A. Tinge, J. O. Hassan, C. Gentry-Weeks, Kelly. SM. Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry. In: L. C. Blankenship, J.H.S. Bailey , N.A. Cox , Stern NJ, Meinersmann RJ, editors. Colonization control of human bacterial enteropathogens in poultry. New York Academic Press 1991. p. 169-98.

Zhang X, Kelly SM, Bollen WS, Curtiss R, III. Characterization and immunogenicity of *Salmonella typhimurium* SL1344 and UK-1 D*crp* and D*cdt* deletion mutants. Infection and immunity. 1997;65(12):5381-7.

Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. Journal of bacteriology. 1951;62(3):293-300.

Sambrook J, Russell DW. Molecular cloning : a laboratory manual. 3rd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2001.

Ehretsmann CP, Carpousis AJ, Krisch HM. Specificity of Escherichia coli endoribonuclease RNase E: in vivo and in vitro analysis of mutants in a bacteriophage T4 mRNA processing site. Genes Dev. 1992;6(1):149-59.

McDowall KJ, Kaberdin VR, Wu SW, Cohen SN, Lin-Chao S. Site-specific RNase E cleavage of oligonucleotides and inhibition by stem-loops. Nature. 1995;374(6519):287-90.

Lin-Chao S, Wong TT, McDowall KJ, Cohen SN. Effects of nucleotide sequence on the specificity of rne-dependent and RNase E-mediated cleavages of RNA I encoded by the pBR322 plasmid. J Biol Chem. 1994;269(14):10797-803.

Galán JE, Nakayama K, Curtiss R, III. Cloning and characterization of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene. 1990;94(1):29-35.

Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. Journal of bacteriology. 1983;154(1):269-77.

Curtiss R, III, Wanda SY, Gunn BM, Zhang X, Tinge SA, Ananthnarayan V, Mo H, Wang S, Kong W. *Salmonella enterica* serovar Typhimurium strains with regulated delayed attenuation in vivo. Infect Immun. 2009;77(3):1071-82.

Shi H, Santander J, Brenneman KE, Wanda SY, Wang S, Senechal P, Sun W, Roland KL, Curtiss R, III. Live recombinant *Salmonella* Typhi vaccines constructed to investigate

the role of *rpoS* in eliciting immunity to a heterologous antigen. PLoS One. 2010;5(6):e11142.

Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, Lorusso A, Vincent AL, Loving CL, Roth JA, Kehrli ME, Jr. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. Vaccine. 2012;30(2):280-8.

Nicholson CO, Costin JM, Rowe DK, Lin L, Jenwitheesuk E, Samudrala R, Isern S, Michael SF. Viral entry inhibitors block dengue antibody-dependent enhancement in vitro. Antiviral Res. 2011;89(1):71-4.

Sasaki T, Setthapramote C, Kurosu T, Nishimura M, Asai A, Omokoko MD, Pipattanaboon C, Pitaksajjakul P, Limkittikul K, Subchareon A, Chaichana P, Okabayashi T, Hirai I, Leaungwutiwong P, Misaki R, Fujiyama K, Ono K, Okuno Y, Ramasoota P, Ikuta K. Dengue virus neutralization and antibody-dependent enhancement activities of human monoclonal antibodies derived from dengue patients at acute phase of secondary infection. Antiviral Res. 2013;98(3):423-31.

Kong W, Brovold M, Koeneman BA, Clark-Curtiss J, and Curtiss III, R. Turning self-destructing *Salmonella* into a uniersal DNA vaccine delivery platform. Proc. Natl. Acad. Sci. USA. 2012: 109(47):19414-19419.

Fluman N, Navon S, Bibi E, Pilpel Y. 2014. mRNA-programmed translation pauses in the targeting of E. coli membrane proteins. Elife 3.

Komar AA, Lesnik T, Reiss C. 1999. Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation. FEBS Lett 462:387-391.

Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM. 2007. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315:525-528.

Cortazzo P, Cervenansky C, Marin M, Reiss C, Ehrlich R, Deana A. 2002. Silent mutations affect in vivo protein folding in Escherichia coli. Biochem Biophys Res Commun 293:537-541.

Gingold H, Pilpel Y. 2011. Determinants of translation efficiency and accuracy. Mol Syst Biol 7:481.

CLAIMS

I claim:

1. A genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen that delivers synthesized ZIKV protein antigens that may, optionally, be encoded by codon optimized nucleotide sequences and/or engineered for translational acceleration or delay, to an immunized animal host.

2. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 1, wherein said genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen exhibits in vivo regulated delayed attenuation, regulated delayed synthesis of protective ZIKV antigens and regulated delayed lysis with delivery of non-glycosylated ZIKV antigens to induce mucosal and systemic antibody responses and protective mucosal and systemic cellular immune responses specific to ZIKV.

3. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 1 or 2, wherein said mucosal and systemic antibody responses and protective mucosal and systemic cellular immune responses are specific to ZIKV and do not cross react with other flaviviruses.

4. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 3, wherein said mucosal and systemic antibody responses and protective mucosal and systemic cellular immune responses are specific to ZIKV and do not cross react with Dengue virus antigens.

5. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claims 1-2, wherein said genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen possesses one or more mutations that enables escape from the pathogen containing vesicle (PSC) into the cytosol for regulated cell lysis for delivery of protein antigens to the proteosome to enhance induction of antigen-specific cellular immune responses.

6. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claims 1-2 in which contains a regulated delayed lysis plasmid vector that encodes ZIKV antigens specified by nucleotide sequences codon-optimized for high-level expression and synthesis of ZIKV protein antigens and/or engineered for translational acceleration or delay.

7. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 2 in which the regulated phenotypes are dependent on the addition of two sugars during growth of the recombinant bacterium.

8. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 7, wherein the two sugars are arabinose and mannose.

9. The bacterium of claims 1-8 in which the ZIKV protein antigen is NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, a NS1-NS2A-NS2B-NS3 polyprotein fusion or a NS4A-2K-NS4B-NS5 polyprotein fusion, any of which may be, optionally, engineered for translational acceleration or delay.

10. The bacterium of claims 1-9, in which the ZIKV protein antigen is capsid (C), pre-membrane (prM) or envelope (E) or a combination of any two or all three antigens, any of which may be engineered for acceleration or translational delay.

11. A genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen that delivers a DNA vaccine vector encoding ZIKV protein antigens, optionally engineered for translational acceleration or delay, to be synthesized in an immunized animal host.

12. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 11 that exhibits in vivo regulated delayed attenuation and regulated delayed lysis occurs in the cytosol to release the DNA vaccine vector for expression in host cells of the immunized animal host to induce mucosal and systemic antibody responses and protective mucosal and systemic cellular immune responses with such immune responses specific to ZIKV and not cross reacting with other flaviviruses.

13. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen of claim 10 that comprises a DNA vaccine vector that encodes ZIKV antigens with modified nucleotide sequences that eliminate N-glycosylation sites and O-glycosylation sites to lead to the synthesis in the immunized animal host of virus-specific antigens that are not glycosylated by post-translational modification.

14. The bacterium of claims 11-13 in which the DNA vaccine vector encodes ZIKV protein antigen capsid (C), pre-membrane (prM) or envelope (E) or a combination of any two or all three antigens which may, optionally, be codon optimized and/or engineered for translational acceleration or delay.

15. The genetically modified recombinant derivative of a facultative intracellular invasive bacterial pathogen according to any preceding claim, wherein said bacterium is a recombinant attenuated *Salmonella* vaccine (RASV) strain is a *Salmonella enterica* serovar, a *S.* Typhimurium strain, *S.* Typhi strain, *S.* Paratyphi A strain, *S.* Gallinarum strain, *S.* Enteritidis strain, *S.* Choleraesuis strain or *S.* Dublin strain.

16. The RASV according to claim 15, said strain further comprising a Δ*pagP*::P_{trc} *lpxE* mutation to reduce toxicity during lysis by synthesis of the non-toxic adjuvant mono-phosphoryl lipid A.

17. A vaccine composition, the composition comprising a recombinant bacterium according to any one of claims 1-14.

18. A method of inducing immunity to ZIKV, the method comprising administering an effective amount of a vaccine composition of claim 17 to an animal host.

19. The method of claim 18, wherein said method induces protective immunity in a subject.

20. The method according to claim 18 or 19, wherein said method induces the production of antibodies that are not substantially cross reactive to a flavivirus antigen.

21. The method of claim 20, wherein said flavivirus is a Dengue virus antigen.

22. The method according to claim 18-19, wherein said immune response produces neutralizing antibodies against ZIKV that do not cross-react with a flavivirus.

23. The method of claim 22, wherein said flavivirus is a Dengue virus antigen.

24. The method of claims 18-19, wherein said method induces mucosal immunity.

25. The method of claim 24, wherein said mucosal immune neutralizes ZIKV found in or on mucosal surfaces of said subject.

26. A recombinant attenuated *Salmonella* vaccine (RASV) strain comprising a nucleic acid sequence encoding a ZIKA virus (ZIKV) antigen selected from glycosylated or non-glycosylated ZIKV capsid (C), membrane precursor (prM), envelope (E) proteins, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 fusion proteins comprising two or more of said glycosylated or non-glycosylated antigens which may, optionally, be codon optimized and/or engineered for translational acceleration or delay, said RASV comprising the following genetic modifications:

a) (ΔP_{murA25}::TT *araC* P_{BAD} *murA;* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ*pmi-2426;* Δ(*wza-wcaM*)*-8* Δ*relA197*::*araC* P_{BAD} *lacI* TT; and Δ*recF126* Δ*sifA26*); or

b) (ΔP_{murA25}::TT *araC* P_{BAD} *murA;* Δ*asdA27*::TT *araC* P_{BAD} *c2;* Δ(*wza-wcaM*)*-8* Δ*pmi-2426;* Δ*relA1123;* Δ*recF126;* Δ*endA2113;* Δ*sseL116;* Δ*tlpA181;*and ΔP_{hilA}::$P_{trc\ \Delta lacO888}$*hilA*).

27. The RASV strain according to claim 26, said RASV strain further comprising a Δ*pagP88*::P_{trc} *lpxE* mutation and expressing.

28. The RASV strain according to claim 26 or 27, wherein said fusion protein comprises NS1-NS2A-NS2B-NS3 or ZIKV NS4A-2K-NS4B-NS5 which may, optionally, be codon-optimized and/or engineered for translational acceleration or delay.

29. The RASV strain according to claim 26 or 27, wherein said RASV strain comprises a nucleic acid sequence encoding a non-glycosylated ZIKA virus (ZIKV) antigen selected from ZIKV capsid (C), membrane precursor (prM), envelope (E) proteins, NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5 or fusion proteins comprising two or more of said non-glycosylated antigens, any of which may, optionally, be codon-optimized and/or engineered for translational acceleration or delay.

30. The RASV strain according to claim 29, wherein said RASV strain comprises a nucleic acid sequence encoding a non-glycosylated E protein.

31. The RASV strain according to claim 30, wherein said nucleic acid sequence encodes non-glycosylated E protein and said nucleic acid sequence comprises a sequence that encodes a mutated glycosylation motif or comprises deletion of said glycosylation motif from said E protein.

32. The RASV strain according to claims 26-31, wherein said RASV strain is a *Salmonella enterica* serovar, a *S.* Typhimurium strain, *S.* Typhi strain, *S.* Paratyphi A strain, *S.* Gallinarum strain, *S.* Enteritidis strain, *S.* Choleraesius strain, or *S.* Dublin strain.

33. A vaccine composition comprising a RASV strain according to any one of claims 26-31 and a pharmaceutically acceptable carrier.

34. The vaccine composition according to claim 33, said vaccine composition further comprising an adjuvant.

35. A method of inducing an immune response in a host comprising the administration of a RASV strain according to any one of claims 26-32 to a host.

36. The method according to claim 35, wherein said immune response produces antibodies that are not substantially cross reactive to Dengue virus antigens.

37. The method according to claim 35, wherein said immune response produces neutralizing antibodies against ZIKV that do not cross-react with DENV.

38. A method of inducing an immune response in a host comprising the administration of a vaccine composition according to any one of claims 33 or 34 to a host.

39. The method according to claim 38, wherein said immune response produces antibodies that are not substantially cross reactive to Dengue virus antigens.

40. The method according to claim 39, wherein said immune response produces neutralizing antibodies against ZIKV that do not cross-react with DENV.

41. The method of any one of claims 35-40, said method inducing mucosal immunity that neutralizes the infectivity of ZIKV.

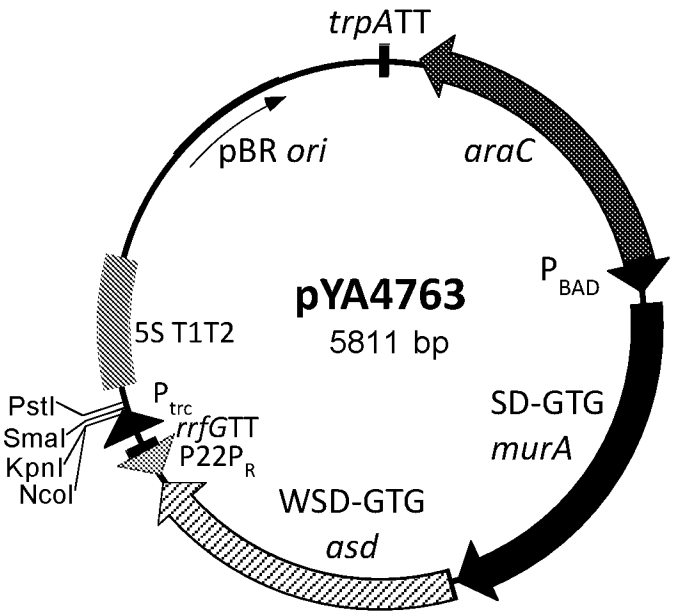

FIGURE 1A

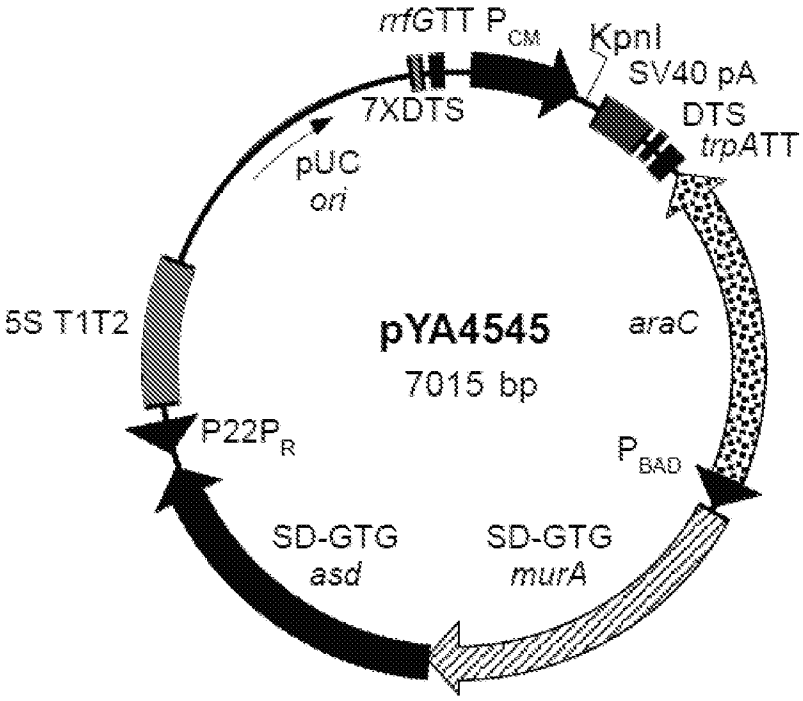

FIGURE 1B

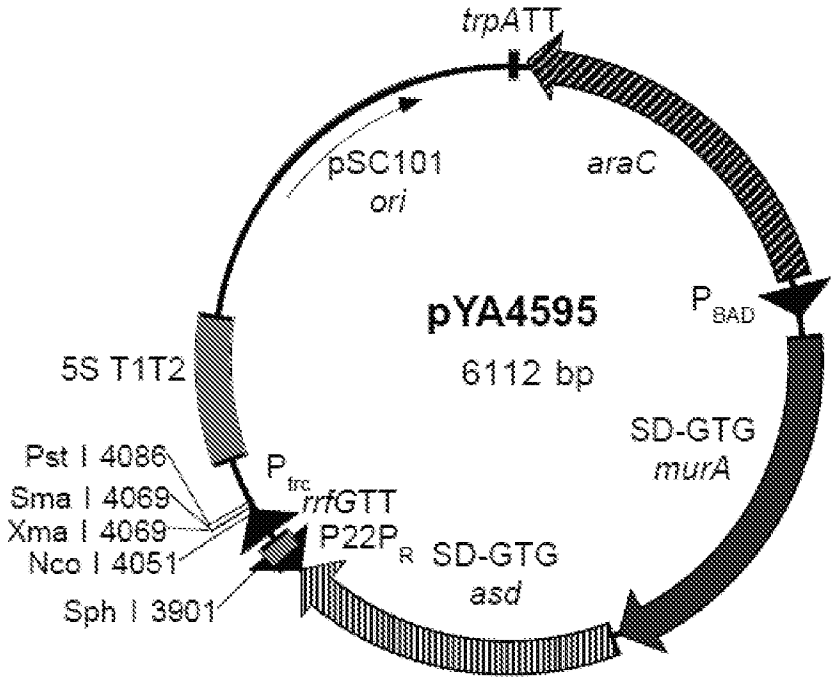

FIGURE 1C

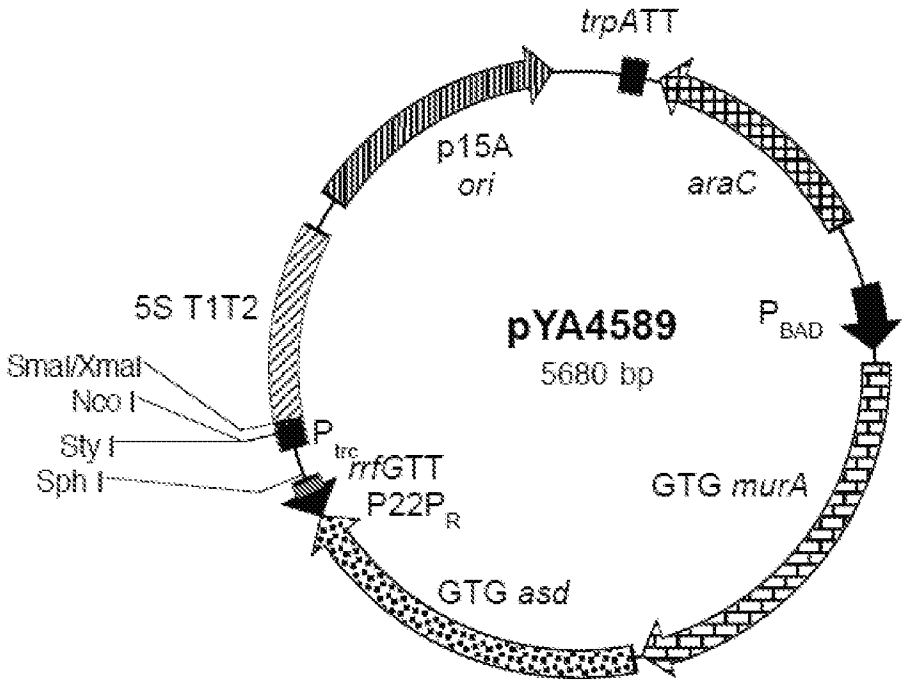

FIGURE 1D

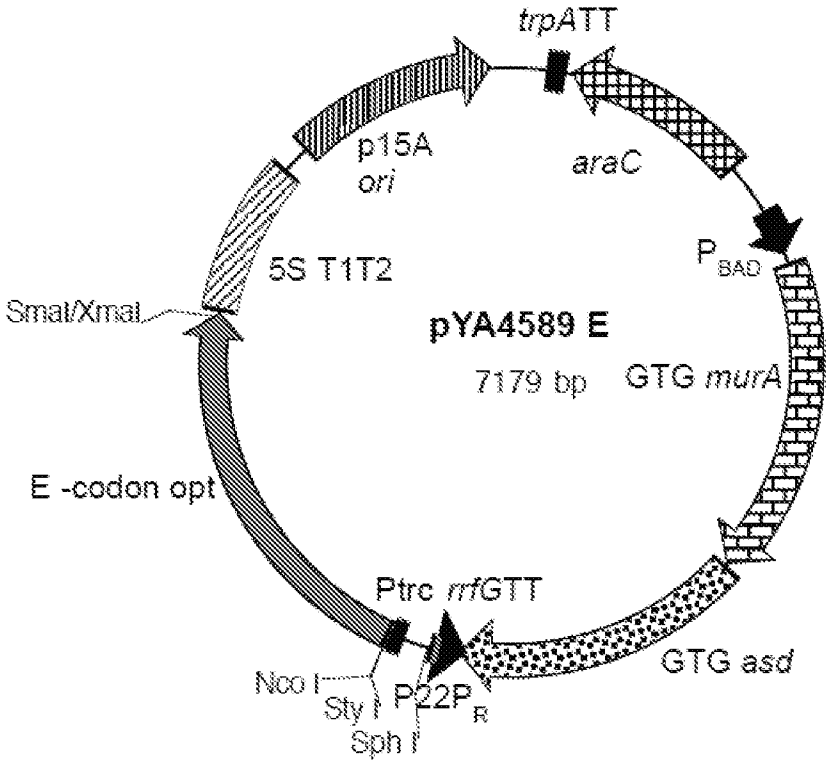

FIGURE 1E

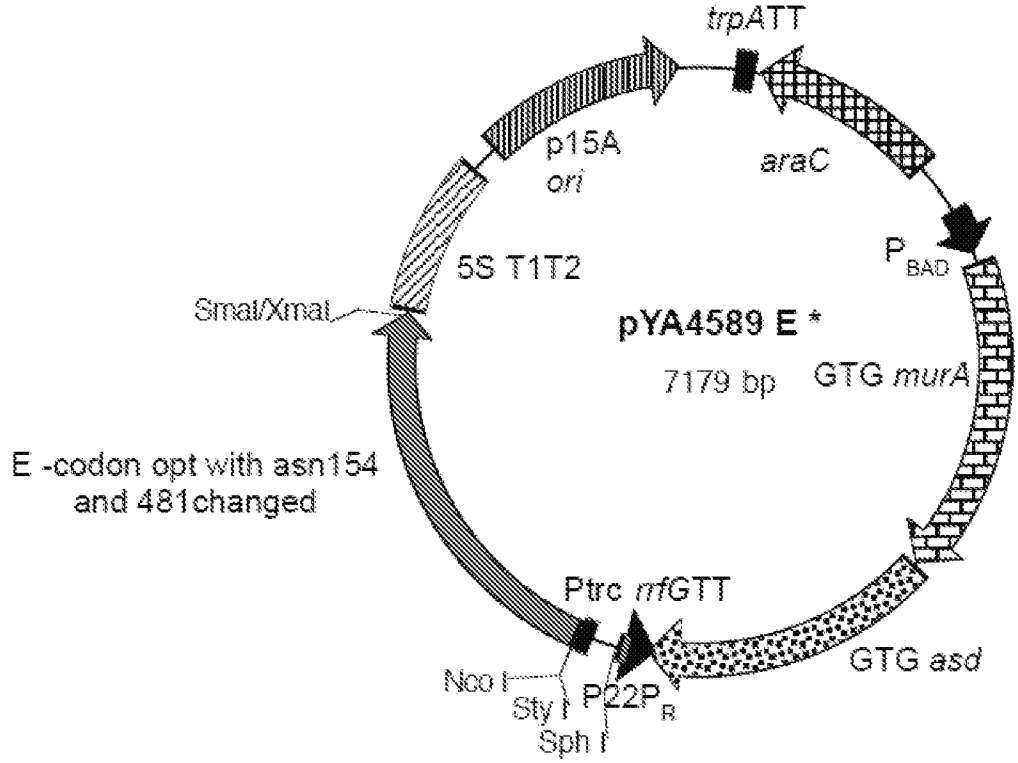

FIGURE 1F

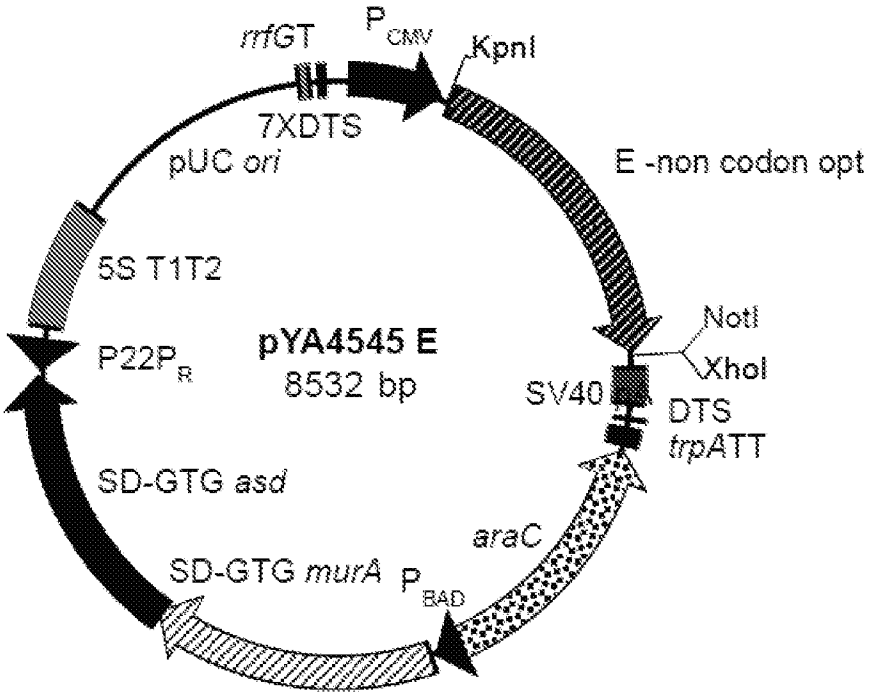

FIGURE 1G

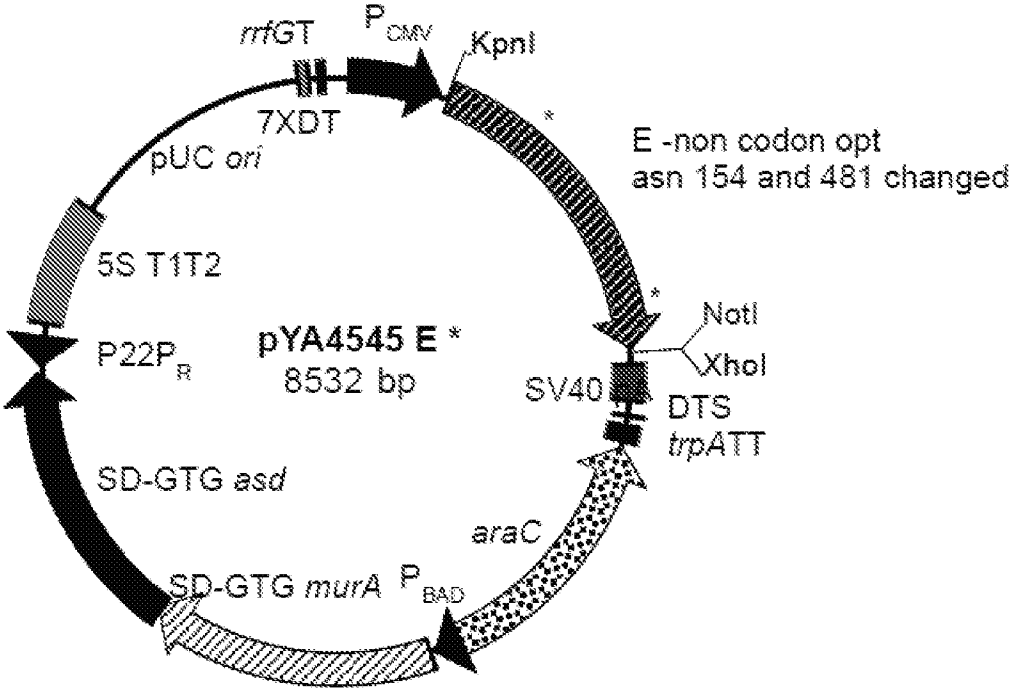

FIGURE 1H

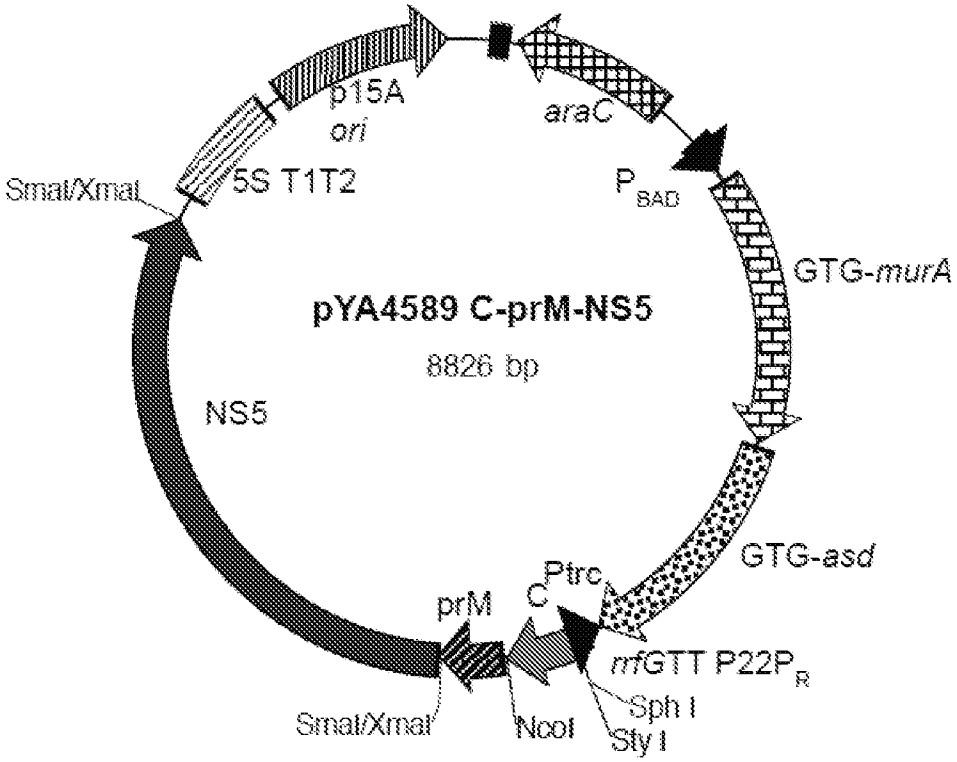
p15A
ori
araC
SmaI/XmaI
5S T1T2
P BAD
GTG-murA
pYA4589 C-prM-NS5
8826 bp
NS5
GTG-asd
Ptrc
C
prM
mfGTT P22P R
SmaI/XmaI
NcoI
Sph I
Sty I

FIGURE 1I

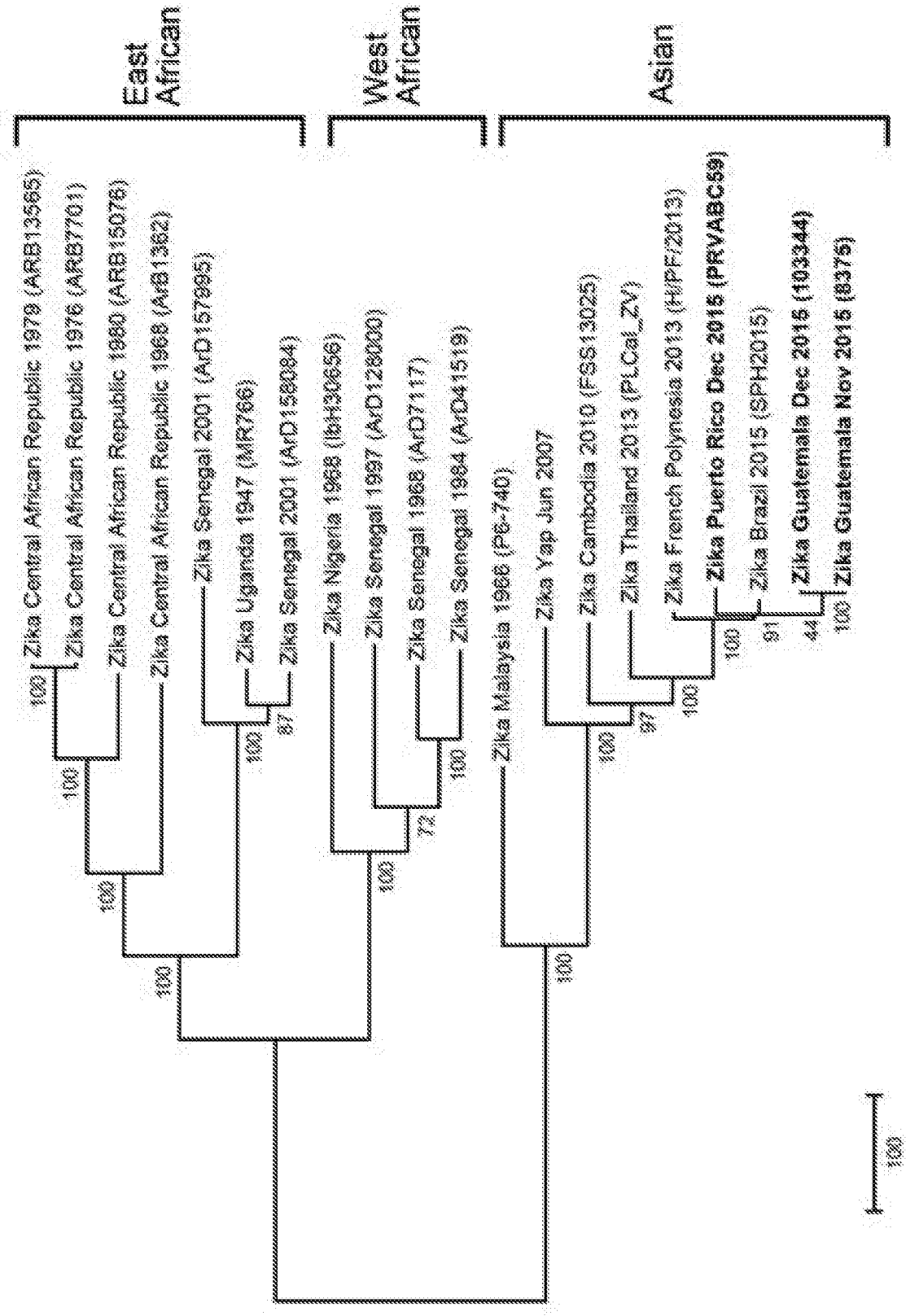
East African
West African
Asian
Zika Central African Republic 1979 (ARB13565)
Zika Central African Republic 1976 (ARB7701)
Zika Central African Republic 1980 (ARB15076)
Zika Central African Republic 1968 (ArB1362)
Zika Senegal 2001 (ArD157995)
Zika Uganda 1947 (MR766)
Zika Senegal 2001 (ArD158084)
Zika Nigeria 1968 (IbH30656)
Zika Senegal 1997 (ArD128000)
Zika Senegal 1968 (ArD7117)
Zika Senegal 1984 (ArD41519)
Zika Malaysia 1966 (P6-740)
Zika Yap Jun 2007
Zika Cambodia 2010 (FSS13025)
Zika Thailand 2013 (PLCal_ZV)
Zika French Polynesia 2013 (H/PF/2013)
Zika Puerto Rico Dec 2015 (PRVABC59)
Zika Brazil 2015 (SPH2015)
Zika Guatemala Dec 2015 (103344)
Zika Guatemala Nov 2015 (8375)
100
100
100
100
100
100
72
100
100
97
100
100
91
44
100
100

FIGURE 2

Capsid protein C 309 bp, (103 aa)

22 aa codon optimized (29 bp changed)

```
1/1                                                 31/11
ATG AAA AAC CCA AAA AAG AAA TCC GGA GGA TTC CGG ATT GTC AAT ATG CTA AAA CGC GGA
ATG AAA AAC CCA AAA AAG AAA TCC GGW GGT TTC CGT ATT GTC AAT ATG CTG AAA CGC GGW
M   K   N   P   K   K   K   S   G   G   F   R   I   V   N   M   L   K   R   G

61/21                                               91/31
GTA GCC CGT GTG AGC CCC TTT GGG GGC TTG AAG AGG CTG CCA GCC GGA CTT CTG CTG GGT
GTA GCC CGT GTG AGC CCG TTT GGC GGC TTG AAG CGT CTG CCA GCC GGC CTG CTG CTG GGT
V   A   R   V   S   P   F   G   G   L   K   R   L   P   A   G   L   L   L   G

121/41                                              151/51
CAT GGG CCC ATC AGG ATG GTC TTG GCG ATT CTA GCC TTT TTG AGA TTC ACG GCA ATC AAG
CAT GGG CCG ATC CGT ATG GTC CTG GCG ATT CTG GCC TTT CTG CGT TTC ACG GCA ATC AAG
H   G   P   I   R   M   V   L   A   I   L   A   F   L   R   F   T   A   I   K

181/61                                              211/71
CCA TCA CTG GGT CTC ATC AAT AGA TGG GGT TCA GTG GGG AAA AAA GAG GCT ATG GAA ACA
CCA TCA CTG GGT CTC ATC AAT CGT TGG GGT TCA GTG GGG AAA AAA GAG GCT ATG GAA ACA
P   S   L   G   L   I   N   R   W   G   S   V   G   K   K   E   A   M   E   T

241/81                                              271/91
ATA AAG AAG TTC AAG AAA GAT CTG GCT GCC ATG CTG AGA ATA ATC AAT GCT AGG AAG GAG
ATC AAG AAG TTC AAG AAA GAT CTG GCT GCC ATG CTG CGC ATT ATC AAT GCT CGC AAG GAG
I   K   K   F   K   K   D   L   A   A   M   L   R   I   I   N   A   R   K   E

301/101
AAG AAG AGA (SEQ ID NO: 1)
AAG AAG CGC (SEQ ID NO: 2)
K   K   R   (SEQ ID NO: 3)
```

FIGURE 3

prM 288 bp (96 aa) optimized and 279 bp (93 aa) original
12 aa (18 bp) codon optimized and 3 N-terminal codons added

```
1/1                                                 31/11
            GCG GAG GTC ACT AGA CGT GGG AGT GCA TAC TAT ATG TAC TTG GAC AGA AAC
ATG AAA AAG GCG GAG GTC ACT CGW CGT GGT AGC GCA TAC TAT ATG TAC CTG GAC CGC AAC
M   K   K   A   E   V   T   R   R   G   S   A   Y   Y   M   Y   L   D   R   N

61/21                                               91/31
GAT GCT GGG GAG GCC ATA TCT TTT CCA ACC ACA TTG GGG ATG AAT AAG TGT TAT ATA CAG
GAT GCT GGC GAG GCC ATC TCT TTT CCA ACC ACC TTG GGT ATG AAT AAG TGT TAT ATC CAG
D   A   G   E   A   I   S   F   P   T   T   L   G   M   N   K   C   Y   I   Q

121/41                                              151/51
ATC ATG GAT CTT GGA CAC ATG TGT GAT GCC ACC ATG AGC TAT GAA TGC CCT ATG CTG GAT
ATC ATG GAT CTS GGC CAC ATG TGT GAT GCC ACC ATG AGC TAT GAA TGC CCT ATG CTG GAT
I   M   D   L   G   H   M   C   D   A   T   M   S   Y   E   C   P   M   L   D


181/61                                              211/71
GAG GGG GTG GAA CCA GAT GAC GTC GAT TGT TGG TGC AAC ACG ACG TCA ACT TGG GTT GTG
GAG GGG GTG GAA CCA GAT GAC GTC GAT TGT TGG TGC AAC ACG ACG TCA ACT TGG GTT GTG
E   G   V   E   P   D   D   V   D   C   W   C   N   T   T   S   T   W   V   V

241/81                                              271/91
TAC GGA ACC TGC CAT CAC AAA AAA GGT GAA GCA CGG AGA TCT AGA AGA (SEQ ID NO: 4)
TAC GGT ACC TGC CAT CAC AAA AAA GGT GAA GCA CGC CGC TCT CGC CGC (SEQ ID NO: 5)
Y   G   T   C   H   H   K   K   G   E   A   R   R   S   R   R   (SEQ ID NO: 6)
```

FIGURE 4

E protein 1518 bp (506 aa) in codon optimized and 1512 bp (504 aa) in original Codon for aa 156(154 in original) and 483 (481 in orginal) changed to cod Q in place of N.54 aa (72 bp) **codon optimized and 2 N-terminal codons added**

```
1/1                                                 31/11
        ATC AGG TGC ATA GGA GTC AGC AAT AGG GAC TTT GTG GAA GGT ATG TCA GGT GGG
ATG AAA ATC CGC TGC ATC GGN GTC AGC AAT CGN GAC TTT GTG GAA GGT ATG TCC GGT GGN
M   K   I   R   C   I   G   V   S   N   R   D   F   V   E   G   M   S   G   G

61/21                                               91/31
ACT TGG GTT GAT GTT GTC TTG GAA CAT GGA GGT TGT GTC ACC GTA ATG GCA CAG GAC AAA
ACT TGG GTT GAT GTT GTC CTG GAA CAT GGC GGT TGT GTC ACC GTA ATG GCA CAG GAC AAA
T   W   V   D   V   V   L   E   H   G   G   C   V   T   V   M   A   Q   D   K

121/41                                              151/51
CCG ACT GTC GAC ATA GAG CTG GTT ACA ACA ACA GTC AGC AAC ATG GCG GAG GTA AGA TCC
CCG ACT GTC GAC ATC GAG CTG GTT ACC ACC ACC GTC AGC AAC ATG GCG GAG GTA CGN TCC
P   T   V   D   I   E   L   V   T   T   T   V   S   N   M   A   E   V   R   S

181/61                                              211/71
TAC TGC TAT GAG GCA TCA ATA TCA GAC ATG GCT TCT GAC AGC CGC TGC CCA ACA CAA GGT
TAC TGC TAT GAG GCA TCC ATC TCC GAC ATG GCT TCT GAC AGC CGC TGC CCA ACC CAA GGT
Y   C   Y   E   A   S   I   S   D   M   A   S   D   S   R   C   P   T   Q   G

241/81                                              271/91
GAA GCC TAC CTT GAC AAG CAA TCA GAC ACT CAA TAT GTC TGC AAA AGA ACG TTA GTG GAC
GAA GCC TAC CTT GAC AAG CAA TCC GAC ACT CAA TAT GTC TGC AAA CGN ACG CTG GTG GAC
E   A   Y   L   D   K   Q   S   D   T   Q   Y   V   C   K   R   T   L   V   D

301/101                                             331/111
AGA GGC TGG GGA AAT GGA TGT GGA CTT TTT GGC AAA GGG AGC CTG GTG ACA TGC GCT AAG
CGN GGC TGG GGC AAT GGN TGT GGC CTT TTT GGT AAA GGG AGC CTG GTG ACC TGC GCT AAG
R   G   W   G   N   G   C   G   L   F   G   K   G   S   L   V   T   C   A   K

361/121                                             391/131
TTT GCA TGC TCC AAG AAA ATG ACC GGG AAG AGC ATC CAG CCA GAG AAT CTG GAG TAC CGG
TTT GCA TGC TCC AAG AAA ATG ACC GGC AAG AGC ATC CAG CCA GAG AAT CTG GAG TAC CGC
F   A   C   S   K   K   M   T   G   K   S   I   Q   P   E   N   L   E   Y   R

421/141                                             451/151
ATA ATG CTG TCA GTT CAT GGC TCC CAG CAC AGT GGG ATG ATC GTT AAT GAC ACA GGA CAT
ATC ATG CTG TCA GTT CAT GGC TCC CAG CAC AGT GGG ATG ATC GTT CAG GAC ACA GGN CAT
I   M   L   S   V   H   G   S   Q   H   S   G   M   I   V   N   D   T   G   H
                                                            Q*
481/161                                             511/171
GAA ACT GAT GAG AAT AGA GCG AAA GTT GAG ATA ACG CCC AAT TCA CCG AGA GCC GAA GCC
GAA ACT GAT GAG AAT CGC GCG AAA GTT GAG ATC ACG CCC AAT TCA CCG CGN GCC GAA GCC
E   T   D   E   N   R   A   K   V   E   I   T   P   N   S   P   R   A   E   A

541/181                                             571/191
ACC CTG GGG GGT TTT GGA AGC CTA GGA CTT GAT TGT GAA CCG AGG ACA GGC CTT GAC TTT
ACC CTG GGG GGT TTT GGT AGC CTA GGT CTT GAT TGT GAA CCG CGN ACA GGC CTT GAC TTT
T   L   G   G   F   G   S   L   G   L   D   C   E   P   R   T   G   L   D   F
```

FIGURE 5

```
601/201                                                 631/211
TCA GAT TTG TAT TAC TTG ACT ATG AAT AAC AAG CAC TGG TTG GTT CAC AAG GAG TGG TTC
TCA GAT TTG TAT TAC TTG ACT ATG AAT AAC AAG CAC TGG TTG GTT CAC AAG GAG TGG TTC
S   D   L   Y   Y   L   T   M   N   N   K   H   W   L   V   H   K   E   W   F

661/221                                                 691/231
CAC GAC ATT CCA TTA CCT TGG CAC GCT GGG GCA GAC ACC GGA ACT CCA CAC TGG AAC AAC
CAC GAC ATT CCA TTA CCT TGG CAC GCT GGG GCA GAC ACC GGW ACT CCA CAC TGG AAC AAC
H   D   I   P   L   P   W   H   A   G   A   D   T   G   T   P   H   W   N   N

721/241                                                 751/251
AAA GAA GCA CTG GTA GAG TTC AAG GAC GCA CAT GCC AAA AGG CAA ACT GTC GTG GTT CTA
AAA GAA GCA CTG GTA GAG TTC AAG GAC GCA CAT GCC AAA CGW CAA ACT GTC GTG GTT CTS
K   E   A   L   V   E   F   K   D   A   H   A   K   R   Q   T   V   V   V   L

781/261                                                 811/271
GGG AGT CAA GAA GGA GCA GTT CAC ACG GCC CTT GCT GGA GCT CTG GAG GCT GAG ATG GAT
GGG AGT CAA GAA GGT GCA GTT CAC ACG GCC CTT GCT GGW GCT CTG GAG GCT GAG ATG GAT
G   S   Q   E   G   A   V   H   T   A   L   A   G   A   L   E   A   E   M   D

841/281                                                 871/291
GGT GCA AAG GGA AGG CTG TCC TCT GGC CAC TTG AAA TGT CGC CTG AAA ATG GAT AAA CTT
GGT GCA AAG GGS CGC CTG TCC TCT GGC CAC TTG AAA TGT CGC CTG AAA ATG GAT AAA CTT
G   A   K   G   R   L   S   S   G   H   L   K   C   R   L   K   M   D   K   L

901/301                                                 931/311
AGA TTG AAG GGC GTG TCA TAC TCC TTG TGT ACT GCA GCG TTC ACA TTC ACC AAG ATC CCG
CGW TTG AAG GGW GTG TCA TAC TCC TTG TGT ACT GCA GCG TTC ACA TTC ACC AAG ATC CCG
R   L   K   G   V   S   Y   S   L   C   T   A   A   F   T   F   T   K   I   P

961/321                                                 991/331
GCT GAA ACA CTG CAC GGG ACA GTC ACA GTG GAG TTA CAG TAC GCA GGG ACA GAT GGA CCT
GCT GAA ACA CTG CAC GGG ACA GTC ACA GTG GAG TTA CAG TAC GCA GGG ACA GAT GGW CCT
A   E   T   L   H   G   T   V   T   V   E   L   Q   Y   A   G   T   D   G   P

1021/341                                                1051/351
TGC AAG GTT CCA GCT CAG ATG GCG GTG GAC ATG CAA ACT CTG ACC CCA GTT GGG AGG TTG
TGC AAG GTT CCA GCT CAG ATG GCG GTG GAC ATG CAA ACT CTG ACC CCA GTT GGG CGW TTG
C   K   V   P   A   Q   M   A   V   D   M   Q   T   L   T   P   V   G   R   L

1081/361                                                1111/371
ATA ACC GCT AAC CCC GTA ATC ACT GAA AGC ACT GAG AAC TCT AAG ATG ATG CTG GAA CTT
ATC ACC GCT AAC CCG GTA ATC ACT GAA AGC ACT GAG AAC TCT AAG ATG ATG CTG GAA CTT
I   T   A   N   P   V   I   T   E   S   T   E   N   S   K   M   M   L   E   L

1141/381                                                1171/391
GAT CCA CCA TTT GGG GAC TCT TAC ATT GTC ATA GGA GTC GGG GAG AAG AAG ATC ACC CAC
GAT CCA CCA TTT GGG GAC TCT TAC ATT GTC ATC GGW GTC GGG GAG AAG AAG ATC ACC CAC
D   P   P   F   G   D   S   Y   I   V   I   G   V   G   E   K   K   I   T   H

1201/401                                                1231/411
CAC TGG CAC AGG AGT GGC AGC ACC ATT GGA AAA GCA TTT GAA GCC ACT GTG AGA GGT GCC
CAC TGG CAC CGS AGT GGC AGC ACC ATT GGW AAA GCA TTT GAA GCC ACT GTG CGW GGT GCC
H   W   H   R   S   G   S   T   I   G   K   A   F   E   A   T   V   R   G   A
```

FIGURE 5 (continued)

```
1261/421                                        1291/431
AAG AGA ATG GCA GTC TTG GGA GAC ACA GCC TGG GAC TTT GGA TCA GTT GGA GGC GCT CTC
AAG CGN ATG GCA GTC TTG GGN GAC ACA GCC TGG GAC TTT GGT TCA GTT GGC GGC GCT CTC
K   R   M   A   V   L   G   D   T   A   W   D   F   G   S   V   G   G   A   L

1321/441                                        1351/451
AAC TCA TTG GGC AAG GGC ATC CAT CAA ATT TTT GGA GCA GCT TTC AAA TCA TTG TTT GGA
AAC TCA TTG GGC AAG GGC ATC CAT CAA ATT TTT GGC GCA GCT TTC AAA TCA TTG TTT GGN

N   S   L   G   K   G   I   H   Q   I   F   G   A   A   F   K   S   L   F   G
1381/461                                        1411/471
GGA ATG TCC TGG TTC TCA CAA ATT CTC ATT GGA ACG TTG CTG ATG TGG TTG GGT CTG AAC
GGN ATG TCC TGG TTC TCA CAA ATT CTC ATT GGA ACG TTG CTG ATG TGG TTG GGT CTG AAC

G   M   S   W   F   S   Q   I   L   I   G   T   L   L   M   W   L   G   L   N
1441/481                                        1471/491
ACA AAG AAT GGA TCT ATT TCC CTT ATG TGC TTG GCC TTA GGG GGA GTG TTG ATC TTC TTA
ACA AAG GAG GGC TCT ATT TCC CTT ATG TGC TTG GCC TTA GGG GGN GTG TTG ATC TTC TTA
T   K   N   G   S   I   S   L   M   C   L   A   L   G   G   V   L   I   F   L
        X*  (SEQ ID NO: 10)
1501/501
TCC ACA GCC GTC TCT GCT (SEQ ID NO: 7)
TCC ACA GCC GTC TCT GCT (SEQ ID NO: 8)
S   T   A   V   S   A   (SEQ ID NO: 9)
```

FIGURE 5 (continued)

NS5 2718 bp (906 aa) in codon optimized and 2709 bp (903 aa) in original 137 aa (196 bp) codon optimized with 3 N-terminl codons added

```
1/1                                             31/11
            GGG GGT GGA ACA GGA GAG ACC CTG GGA GAG AAA TGG AAG GCC CGC TTG AAC
ATG AAA AAG GGT GGT GGN ACC GGN GAG ACC CTG GGN GAG AAA TGG AAG GCC CGC CTG AAC
M   K   K   G   G   G   T   G   E   T   L   G   E   K   W   K   A   R   L   N

61/21                                           91/31
CAG ATG TCG GCC CTG GAG TTC TAC TCC TAC AAA AAG TCA GGC ATC ACC GAG GTG TGC AGA
CAG ATG TCG GCC CTG GAG TTC TAC TCC TAC AAA AAG TCC GGN ATC ACC GAG GTG TGC CGN
Q   M   S   A   L   E   F   Y   S   Y   K   K   S   G   I   T   E   V   C   R

121/41                                          151/51
GAA GAG GCC CGC CGC GCC CTC AAG GAC GGT GTG GCA ACG GGA GGC CAT GCT GTG TCC CGA
GAA GAG GCC CGC CGC GCC CTG AAG GAC GGT GTG GCA ACG GGN GGN CAT GCT GTG TCC CGN
E   E   A   R   R   A   L   K   D   G   V   A   T   G   G   H   A   V   S   R

181/61                                          211/71
GGA AGT GCA AAG CTG AGA TGG TTG GTG GAG CGG GGA TAC CTG CAG CCC TAT GGA AAG GTC
GGN AGC GCA AAG CTG CGN TGG CTG GTG GAG CGG GGN TAC CTG CAG CCN TAT GGN AAG GTC
G   S   A   K   L   R   W   L   V   E   R   G   Y   L   Q   P   Y   G   K   V
```

FIGURE 6

241/81 271/91
ATT GAT CTT GGA TGT GGC AGA GGG GGC TGG AGT TAC TAC GTC GCC ACC ATC CGC AAA GTT
ATT GAT CTT GGW TGT GGC CGW GGC GGC TGG AGC TAC TAC GTC GCC ACC ATC CGC AAA GTT
I D L G C G R G G W S Y Y V A T I R K V

301/101 331/111
CAA GAA GTG AAA GGA TAC ACA AAA GGA GGC CCT GGT CAT GAA GAA CCC GTG TTG GTG CAA
CAA GAA GTG AAA GGT TAC ACC AAA GGW GGC CCT GGT CAT GAA GAA CCM GTG CTG GTG CAA
Q E V K G Y T K G G P G H E E P V L V Q

361/121 391/131
AGC TAT GGG TGG AAC ATA GTC CGT CTT AAG AGT GGG GTG GAC GTC TTT CAT ATG GCG GCT
AGC TAT GGC TGG AAC ATW GTC CGT CTS AAG AGC GGC GTG GAC GTC TTT CAT ATG GCG GCT
S Y G W N I V R L K S G V D V F H M A A

421/141 451/151
GAG CCG TGT GAC ACG TTG CTG TGT GAC ATA GGT GAG TCA TCA TCT AGT CCT GAA GTG GAA
GAG CCG TGT GAC ACG CTG CTG TGT GAC ATW GGT GAG TCC TCC TCT AGT CCM GAA GTG GAA
E P C D T L L C D I G E S S S S P E V E

481/161 511/171
GAA GCA CGG ACG CTC AGA GTC CTC TCC ATG GTG GGG GAT TGG CTT GAA AAA AGA CCA GGA
GAA GCA CGW ACG CTC CGW GTC CTG TCC ATG GTG GGW GAT TGG CTS GAA AAA CGW CCA GGW
E A R T L R V L S M V G D W L E K R P G

541/181 571/191
GCC TTT TGT ATA AAA GTG TTG TGC CCA TAC ACC AGC ACT ATG ATG GAA ACC CTG GAG CGA
GCC TTT TGT ATW AAA GTG CTG TGC CCA TAC ACC AGC ACT ATG ATG GAA ACC CTG GAG CGW
A F C I K V L C P Y T S T M M E T L E R

601/201 631/211
CTG CAG CGT AGG TAT GGG GGA GGA CTG GTC AGA GTG CCA CTC TCC CGC AAC TCT ACA CAT
CTG CAG CGT CGW TAT GGG GGW GGW CTG GTC CGT GTG CCA CTC TCC CGC AAC TCT ACA CAT
L Q R R Y G G G L V R V P L S R N S T H

661/221 691/231
GAG ATG TAC TGG GTC TCT GGA GCG AAA AGC AAC ACC ATA AAA AGT GTG TCC ACC ACG AGC
GAG ATG TAC TGG GTC TCT GGW GCG AAA AGC AAC ACC ATW AAA AGT GTG TCC ACC ACG AGC
E M Y W V S G A K S N T I K S V S T T S

721/241 751/251
CAG CTC CTC TTG GGG CGC ATG GAC GGG CCT AGG AGG CCA GTG AAA TAT GAG GAG GAT GTG
CAG CTC CTC TTG GGG CGC ATG GAC GGG CCT CGW CGT CCA GTG AAA TAT GAG GAG GAT GTG
Q L L L G R M D G P R R P V K Y E E D V

781/261 811/271
AAT CTC GGC TCT GGC ACG CGG GCT GTG GTA AGC TGC GCT GAA GCT CCC AAC ATG AAG ATC
AAT CTC GGT TCT GGW ACG CGW GCT GTG GTA AGC TGC GCT GAA GCT CCM AAC ATG AAG ATC
N L G S G T R A V V S C A E A P N M K I

841/281 871/291
ATT GGT AAC CGC ATT GAA AGG ATC CGC AGT GAG CAC GCG GAA ACG TGG TTC TTT GAC GAG
ATT GGT AAC CGC ATT GAA CGW ATC CGC AGT GAG CAC GCG GAA ACG TGG TTC TTT GAC GAG
I G N R I E R I R S E H A E T W F F D E

FIGURE 6 (continued)

```
901/301                                                 931/311
AAC CAC CCA TAT AGG ACA TGG GCT TAC CAT GGA AGC TAT GAG GCC CCC ACA CAA GGG TCA
AAC CAC CCA TAT CGT ACA TGG GCT TAC CAT GGT AGC TAT GAG GCC CCA ACA CAA GGG TCA
N   H   P   Y   R   T   W   A   Y   H   G   S   Y   E   A   P   T   Q   G   S

961/321                                                 991/331
GCG TCC TCT CTA ATA AAC GGG GTT GTC AGG CTC CTG TCA AAA CCC TGG GAT GTG GTG ACT
GCG TCC TCT CTG ATT AAC GGG GTT GTC CGT CTC CTG TCA AAA CCA TGG GAT GTG GTG ACT
A   S   S   L   I   N   G   V   V   R   L   L   S   K   P   W   D   V   V   T

1021/341                                                1051/351
GGA GTC ACA GGA ATA GCC ATG ACC GAC ACC ACA CCG TAT GGT CAG CAA AGA GTT TTC AAG
GGT GTC ACA GGN ATT GCC ATG ACC GAC ACC ACA CCG TAT GGT CAG CAA CGT GTT TTC AAG
G   V   T   G   I   A   M   T   D   T   T   P   Y   G   Q   Q   R   V   F   K

1081/361                                                1111/371
GAA AAA GTG GAC ACT AGG GTG CCA GAC CCC CAA GAA GGC ACT CGT CAG GTT ATG AGC ATG
GAA AAA GTG GAC ACT CGT GTG CCA GAC CCA CAA GAA GGC ACT CGT CAG GTT ATG AGC ATG
E   K   V   D   T   R   V   P   D   P   Q   E   G   T   R   Q   V   M   S   M

1141/381                                                1171/391
GTC TCT TCC TGG TTG TGG AAA GAG CTA GGC AAA CAC AAA CGG CCA CGA GTC TGC ACC AAA
GTC TCT TCC TGG TTG TGG AAA GAG CTG GGC AAA CAC AAA CGT CCA CGT GTC TGC ACC AAA
V   S   S   W   L   W   K   E   L   G   K   H   K   R   P   R   V   C   T   K

1201/401                                                1231/411
GAA GAG TTC ATC AAC AAG GTT CGT AGC AAT GCA GCA TTA GGG GCA ATA TTT GAA GAG GAA
GAA GAG TTC ATC AAC AAG GTT CGT AGC AAT GCA GCA TTA GGG GCA ATT TTT GAA GAG GAA
E   E   F   I   N   K   V   R   S   N   A   A   L   G   A   I   F   E   E   E

1261/421                                                1291/431
AAA GAG TGG AAG ACT GCA GTG GAA GCT GTG AAC GAT CCA AGG TTC TGG GCT CTA GTG GAC
AAA GAG TGG AAG ACT GCA GTG GAA GCT GTG AAC GAT CCA CGT TTC TGG GCT CTG GTG GAC
K   E   W   K   T   A   V   E   A   V   N   D   P   R   F   W   A   L   V   D

1321/441                                                1351/451
AAG GAA AGA GAG CAC CAC CTG AGA GGA GAG TGC CAG AGC TGT GTG TAC AAC ATG ATG GGA
AAG GAA CGT GAG CAC CAC CTG CGT GGT GAG TGC CAG AGC TGT GTG TAC AAC ATG ATG GGT
K   E   R   E   H   H   L   R   G   E   C   Q   S   C   V   Y   N   M   M   G

1381/461                                                1411/471
AAA AGA GAA AAG AAA CAA GGG GAA TTT GGA AAG GCC AAG GGC AGC CGC GCC ATC TGG TAT
AAA CGT GAA AAG AAA CAA GGG GAA TTT GGT AAG GCC AAG GGC AGC CGC GCC ATC TGG TAT
K   R   E   K   K   Q   G   E   F   G   K   A   K   G   S   R   A   I   W   Y

1441/481                                                1471/491
ATG TGG CTA GGG GCT AGA TTT CTA GAG TTC GAA GCC CTT GGA TTC TTG AAC GAG GAT CAC
ATG TGG CTG GGG GCT CGT TTT CTG GAG TTC GAA GCC CTT GGT TTC TTG AAC GAG GAT CAC
M   W   L   G   A   R   F   L   E   F   E   A   L   G   F   L   N   E   D   H

1501/501                                                1531/511
TGG ATG GGG AGA GAG AAC TCA GGA GGT GGT GTT GAA GGG CTG GGA TTA CAA AGA CTC GGA
TGG ATG GGG CTG GAG AAC TCA GGT GGT GGT GTT GAA GGG CTG GGT TTA CAA CGT CTC GGT
W   M   G   R   E   N   S   G   G   G   V   E   G   L   G   L   Q   R   L   G
```

FIGURE 6 (continued)

1561/521 1591/531
TAT GTC CTA GAA GAG ATG AGT CGT ATA CCA GGA GGA AGG ATG TAT GCA GAT GAC ACT GCT
TAT GTC CTS GAA GAG ATG AGT CGT ATY CCA GGY GGY CGY ATG TAT GCA GAT GAC ACT GCT
Y V L E E M S R I P G G R M Y A D D T A

1621/541 1651/551
GGC TGG GAC ACC CGC ATT AGC AGG TTT GAT CTG GAG AAT GAA GCT CTA ATC ACC AAC CAA
GGC TGG GAC ACC CGC ATT AGC CGY TTT GAT CTG GAG AAT GAA GCT CTS ATC ACC AAC CAA
G W D T R I S R F D L E N E A L I T N Q

1681/561 1711/571
ATG GAG AAA GGG CAC AGG GCC TTG GCA TTG GCC ATA ATC AAG TAC ACA TAC CAA AAC AAA
ATG GAG AAA GGG CAC CTS GCC TTG GCA TTG GCC ATY ATC AAG TAC ACA TAC CAA AAC AAA
M E K G H R A L A L A I I K Y T Y Q N K

1741/581 1771/591
GTG GTA AAG GTC CTT AGA CCA GCT GAA AAA GGG AAA ACA GTT ATG GAC ATT ATT TCG AGA
GTG GTA AAG GTC CTT CGY CCA GCT GAA AAA GGG AAA ACA GTT ATG GAC ATT ATT TCG CGY
V V K V L R P A E K G K T V M D I I S R

1801/601 1831/611
CAA GAC CAA AGG GGG AGC GGA CAA GTT GTC ACT TAC GCT CTT AAC ACA TTT ACC AAC CTA
CAA GAC CAA CGY GGG AGC GGY CAA GTT GTC ACT TAC GCT CTT AAC ACA TTT ACC AAC CTS
Q D Q R G S G Q V V T Y A L N T F T N L

1861/621 1891/631
GTG GTG CAA CTC ATT CGG AAT ATG GAG GCT GAG GAA GTT CTA GAG ATG CAA GAC TTG TGG
GTG GTG CAA CTC ATT CGY AAT ATG GAG GCT GAG GAA GTT CTS GAG ATG CAA GAC TTG TGG
V V Q L I R N M E A E E V L E M Q D L W

1921/641 1951/651
CTG CTG CGG AGG TCA GAG AAA GTG ACC AAC TGG TTG CAG AGC AAC GGA TGG GAT AGG CTC
CTG CTG CGY CGY TCA GAG AAA GTG ACC AAC TGG TTG CAG AGC AAC GGY TGG GAT **C**GY CTC
L L R R S E K V T N W L Q S N G W D R L

1981/661 2011/671
AAA CGA ATG GCA GTC AGT GGA GAT GAT TGC GTT GTG AAG CCA ATT GAT GAT AGG TTT GCA
AAA CGY ATG GCA GTC AGT GGY GAT GAT TGC GTT GTG AAG CCA ATT GAT GAT CGY TTT GCA
K R M A V S G D D C V V K P I D D R F A

2041/681 2071/691
CAT GCC CTC AGG TTC TTG AAT GAT ATG GGA AAA GTT AGG AAG GAC ACA CAA GAG TGG AAA
CAT GCC CTC CGY TTC TTG AAT GAT ATG GGY AAA GTT CGY AAG GAC ACA CAA GAG TGG AAA
H A L R F L N D M G K V R K D T Q E W K

2101/701 2131/711
CCC TCA ACT GGA TGG GAC AAC TGG GAA GAA GTT CCG TTT TGC TCC CAC CAC TTC AAC AAG
CCM TCA ACT GGY TGG GAC AAC TGG GAA GAA GTT CCG TTT TGC TCC CAC CAC TTC AAC AAG
P S T G W D N W E E V P F C S H H F N K

2161/721 2191/731
CTC CAT CTC AAG GAC GGG AGG TCC ATT GTG GTT CCC TGC CGC CAC CAA GAT GAA CTG ATT
CTC CAT CTC AAG GAC GGG CGY TCC ATT GTG GTT CCM TGC CGC CAC CAA GAT GAA CTG ATT
L H L K D G R S I V V P C R H Q D E L I

FIGURE 6 (continued)

```
2221/741                                    2251/751
GGC CGG GCC CGC GTC TCT CCA GGG GCG GGA TGG AGC ATC CGG GAG ACT GCT TGC CTA GCA
GGC CGN GCC CGC GTC TCT CCA GGG GCG GGN TGG AGC ATC CGN GAG ACT GCT TGC CTN GCA
G   R   A   R   V   S   P   G   A   G   W   S   I   R   E   T   A   C   L   A

2281/761                                    2311/771
AAA TCA TAT GCG CAA ATG TGG CAG CTC CTT TAT TTC CAC AGA AGG GAC CTC CGA CTG ATG
AAA TCA TAT GCG CAA ATG TGG CAG CTC CTT TAT TTC CAC CGN CGN GAC CTC CGN CTG ATG
K   S   Y   A   Q   M   W   Q   L   L   Y   F   H   R   R   D   L   R   L   M

2341/781                                    2371/791
GCC AAT GCC ATT TGT TCA TCT GTG CCA GTT GAC TGG GTT CCA ACT GGG AGA ACT ACC TGG
GCC AAT GCC ATT TGT TCA TCT GTG CCA GTT GAC TGG GTT CCA ACT GGG CGN ACT ACC TGG
A   N   A   I   C   S   S   V   P   V   D   W   V   P   T   G   R   T   T   W

2401/801                                    2431/811
TCA ATC CAT GGA AAG GGA GAA TGG ATG ACC ACT GAA GAC ATG CTT GTG GTG TGG AAC AGA
TCA ATC CAT GGN AAG GGN GAA TGG ATG ACC ACT GAA GAC ATG CTT GTG GTG TGG AAC CGN
S   I   H   G   K   G   E   W   M   T   T   E   D   M   L   V   V   W   N   R

2461/821                                    2491/831
GTG TGG ATT GAG GAG AAC GAC CAC ATG GAA GAC AAG ACC CCA GTT ACG AAA TGG ACA GAC
GTG TGG ATT GAG GAG AAC GAC CAC ATG GAA GAC AAG ACC CCA GTT ACG AAA TGG ACA GAC
V   W   I   E   E   N   D   H   M   E   D   K   T   P   V   T   K   W   T   D

2521/841                                    2551/851
ATT CCC TAT TTG GGA AAA AGG GAA GAC TTG TGG TGT GGA TCT CTC ATA GGG CAC AGA CCG
ATT CCN TAT TTG GGN AAA CGN GAA GAC TTG TGG TGT GGN TCT CTC ATN GGG CAC CGN CCG
I   P   Y   L   G   K   R   E   D   L   W   C   G   S   L   I   G   H   R   P

2581/861                                    2611/871
CGC ACC ACC TGG GCT GAG AAC ATT AAA AAC ACA GTC AAC ATG GTG CGC AGG ATC ATA GGT
CGC ACC ACC TGG GCT GAG AAC ATT AAA AAC ACA GTC AAC ATG GTG CGC CGN ATC ATT GGT
R   T   T   W   A   E   N   I   K   N   T   V   N   M   V   R   R   I   I   G

2641/881                                    2671/891
GAT GAA GAA AAG TAC ATG GAC TAC CTA TCC ACC CAA GTT CGC TAC TTG GGT GAA GAA GGG
GAT GAA GAA AAG TAC ATG GAC TAC CTN TCC ACC CAA GTT CGC TAC TTG GGT GAA GAA GGG
D   E   E   K   Y   M   D   Y   L   S   T   Q   V   R   Y   L   G   E   E   G
                                                                (SEQ ID NO: 13)

2701/901
TCT ACA CCT GGA GTG CTG (SEQ ID NO: 11)
TCT ACA CCT GGN GTG CTG (SEQ ID NO: 12)
```

FIGURE 6 (continued)

GACTCTTCGCGATGTACGGGCCAGATATACGCGTTAACTGCAGTCTAGAT
TATGCGAAAGGCCATCCTGACGGATGGCCTTTTTGTTTAAACGGATCCGC
GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTA
GgggactttccggggactttccTccccacgcgGgggactttccgccacgg
gcggggactttccggggactttccGTTCATAGCCCATATATGGAGTTCCG
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC
CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATA
GGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCA
CTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACG
TCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
CATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG
ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTG
TTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGC
CCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAA
GCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAA
ATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTT
AAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCTGC
AGATATCCAGCACAGTGGCGGCCGCTCGAGAATGCTTCGAGCAGACATGA
TAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAA
AAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCAT
TATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGT
TTCAGGTTCAGGGGGAGATGTGGGAGGTTTTTTAAAGCAAGTAAAACCTC
TACAAATGTGGTAAAATCCGATAAGGATCGATCCGGGGCATGCAACCAGC
TGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCA
GGCAGAAGTATGCAAAGCATGTGGGGATGCGGTGGGCTCTATGGCTTCTA
CTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGC
CCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTCG
CCGCCAAGGATCTGTCGACCCCTAGATTTCAGTGCAATTTATCTCTTCAA
ATGTAGCACCTGAAGTCAGCCCCATACGATATAAGTTGTTGGAAGATCTA
GCCCGCCTAATGAGCGGGCTTTTTTTTAATTCGCAATTCCCCGATGCATA
ATGTGCCTGTCAAATGGACGAAGCAGGGATTCTGCAAACCCTATGCTACT
CCGTCAAGCCGTCAATTGTCTGATTCGTTACCAATTATGACAACTTGACG
GCTACATCATTCACTTTTTCTTCACAACCGGCACGAAACTCGCTCGGGCT
GGCCCCGGTGCATTTTTTAAATACTCGCGAGAAATAGAGTTGATCGTCAA
AACCAACATTGCGACCGACGGTGGCGATAGGCATCCGGGTAGTGCTCAAA
AGCAGCTTCGCCTGACTAATGCGTTGGTCCTCGCGCCAGCTTAAGACGCT
AATCCCTAACTGCTGGCGGAAAAGATGTGACAGACGCGACGGCGACAAGC

FIGURE 7

AAACATGCTGTGCGACGCTGGCGATATCAAAATTGCTGTCTGCCAGGTGA
TCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCCATCGGTGGATG
GAGCGACTCGTTAATCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGCA
GATTTATCGCCAGCAGCTCCGAATAGCGCCCTTCCCCTTGCCCGGCGTTA
ATGATTTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGG
GCGAAAGAAACCCGTATTGGCAAATATTGACGGCCAGTTAAGCCATTCAT
GCCAGTAGGCGCGCGGACGAAAGTAAACCCACTGGTGATACCATTCGCGA
GCCTCCGGATGACGACCGTAGTGATGAATCTCTCCTGGCGGGAACAGCAA
AATATCACCCGGTCGGCAGACAAATTCTCGTCCCTGATTTTTCACCACCC
CCTGACCGCGAATGGTGAGATTGAGAATATAACCTTTCATTCCCAGCGGT
CGGTCGATAAAAAAATCGAGATAACCGTTGGCCTCAATCGGCGTTAAACC
CGCCACCAGATGGGCGTTAAACGAGTATCCCGGCAGCAGGGGATCATTTT
GCGCTTCAGCCATACTTTTCATACTCCCACCATTCAGAGAAGAAACCAAT
TGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTT
CTCGCTAACCCAACCGGTAACCCCGCTTATTAAAAGCATTCTGTAACAAA
GCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAATCAC
GGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC
CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGACGCTTTTTAT
CGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGGCTAGCGAATTC
TGAGAACAAACTAAGTGGATAAATTTCGTGTTCAGGGGCCAACGAAGCTC
CAGGGCGAAGTCACAATTTCCGGCGCTAAAAATGCTGCTCTGCCTATCCT
TTTTGCCGCACTACTGGCGGAAGAACCGGTAGAGATCCAGAACGTCCCGA
AACTGAAAGACGTCGATACATCAATGAAGCTGCTAAGCCAGCTGGGTGCG
AAAGTAGAACGTAATGGTTCTGTGCATATTGATGCCCGCGACGTTAATGT
ATTCTGCGCACCTTACGATCTGGTTAAAACCATGCGTGCTTCTATCTGGG
CGCTGGGGCCGCTGGTAGCGCGCTTTGGTCAGGGGCAAGTTTCACTACCT
GGCGGTTGTACGATCGGTGCGCGTCCGGTTGATCTACACATTTCTGGCCT
CGAACAATTAGGCGCGACCATCAAACTGGAAGAAGGTTACGTTAAAGCTT
CCGTCGATGGTCGTTTGAAAGGTGCACATATCGTGATGGATAAAGTCAGC
GTTGGCGCAACGGTGACCATCATGTGTGCTGCAACCCTGGCGGAAGGCAC
CACGATTATTGAAAACGCAGCGCGTGAACCGGAAATCGTCGATACCGCGA
ACTTCCTGATTACGCTGGGTGCGAAAATTAGCGGTCAGGGCACCGATCGT
ATCGTCATCGAAGGTGTGGAACGTTTAGGCGGCGGTGTCTATCGCGTTCT
GCCGGATCGTATCGAAACCGGTACTTTCCTGGTGGCGGCGGCGATTTCTC
GCGGCAAAATTATCTGCCGTAACGCGCAGCCAGATACTCTCGACGCCGTG
CTGGCGAAACTGCGTGACGCTGGAGCGGACATCGAAGTCGGCGAAGACTG
GATTAGCCTGGATATGCATGGCAAACGTCCGAAGGCTGTTAACGTACGTA
CCGCGCCGCATCCGGCATTCCCGACCGATATGCAGGCCCAGTTCACGCTG

FIGURE 7 (continued)

TTGAACCTGGTGGCAGAAGGGACCGGGTTTATCACCGAAACGGTCTTTGA
AAACCGCTTTATGCATGTGCCAGAGCTGAGCCGTATGGGCGCGCACGCCG
AAATCGAAAGCAATACCGTTATTTGTCACGGTGTTGAAAAACTTTCTGGC
GCACAGGTTATGGCAACCGATCTGCGTGCATCAGCAAGCCTGGTGCTGGC
TGGCTGTATTGCGGAAGGGACGACGGTGGTTGATCGTATTTATCACATCG
ATCGTGGCTACGAACGCATTGAAGACAAACTGCGCGCTTTAGGTGCAAAT
ATTGAGCGTGTGAAAGGCGAATAAGAATTCAGGAAAAAAACGCTGTGAAA
AATGTTGGTTTTATCGGCTGGCGCGGAATGGTCGGCTCTGTTCTCATGCA
ACGCATGGTAGAGGAGCGCGATTTCGACGCTATTCGCCCTGTTTTCTTTT
CTACCTCCCAGTTTGGACAGGCGGCGCCCACCTTCGGCGACACCTCCACC
GGCACGCTACAGGACGCTTTTGATCTGGATGCGCTAAAAGCGCTCGATAT
CATCGTGACCTGCCAGGGCGGCGATTATACCAACGAAATTTATCCAAAGC
TGCGCGAAAGCGGATGGCAGGGTTACTGGATTGATGCGGCTTCTACGCTG
CGCATGAAAGATGATGCCATTATTATTCTCGACCCGGTCAACCAGGACGT
GATTACCGACGGCCTGAACAATGGCGTGAAGACCTTTGTGGGCGGTAACT
GTACCGTTAGCCTGATGTTGATGTCGCTGGGCGGTCTCTTTGCCCATAAT
CTCGTTGACTGGGTATCCGTCGCGACCTATCAGGCCGCCTCCGGCGGCGG
CGCGCGCCATATGCGCGAGCTGTTAACCCAGATGGGTCAGTTGTATGGCC
ATGTCGCCGATGAACTGGCGACGCCGTCTTCCGCAATTCTTGATATTGAA
CGCAAAGTTACGGCATTGACCCGCAGCGGCGAGCTGCCGGTTGATAACTT
TGGCGTACCGCTGGCGGGAAGCCTGATCCCCTGGATCGACAAACAGCTCG
ATAACGGCCAGAGCCGCGAAGAGTGGAAAGGCCAGGCGGAAACCAACAAG
ATTCTCAATACTGCCTCTGTGATTCCGGTTGATGGTTTGTGTGTGCGCGT
CGGCGCGCTGCGCTGTCACAGCCAGGCGTTCACCATCAAGCTGAAAAAAG
AGGTATCCATTCCGACGGTGGAAGAACTGCTGGCGGCACATAATCCGTGG
GCGAAAGTGGTGCCGAACGATCGTGATATCACTATGCGCGAATTAACCCC
GGCGGCGGTGACCGGCACGTTGACTACGCCGGTTGGTCGTCTGCGTAAGC
TGAACATGGGGCCAGAGTTCTTGTCGGCGTTTACCGTAGGCGACCAGTTG
TTATGGGGCGCCGCCGAGCCGCTGCGTCGAATGCTGCGCCAGTTGGCGTA
GTCTAGCTGCACGATACCGTCGACTTGTACATAGACTCGCTCCGAAATTA
AAGAACACTTAAATTATCTACTAAAGGAATCTTTAGTCAAGTTTATTTAA
GATGACTTAACTATGAATACACAATTGATGGGTGAGCGTAGGATCTTCCA
TTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCTTGGCTGTTTTGG
CGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAA
GCGGTCTGATAAAACAGTTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACC
TGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTG
TGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACG

FIGURE 7 (continued)

AAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGG
TGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTT
GCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGC
CAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT
TTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTATC
CGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATGGAAGATCTTC
CAACATCACAGGTAAACAGAAACGTCGGGTCGATCGGGAAATTCTTTCCC
GGACGGCGCGGGGTTGGGCAAGCCGCAGGCGCGTCAGTGCTTTTAGCGGG
TGTCGGGGCAGCCCTGAACCAGTCACGGGATCGATCTGTGCGGTATTTCA
CACCGCATACAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATT
TGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATA
ACCCTGATAAATGCTTCAATAATAGCACGTGCTAAAACTTCATTTTTAAT
TTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATC
CCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGAT
CAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGC
AAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAG
CTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACC
AAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT
CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCT
GCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATA
GTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACAC
AGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGT
GAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTA
TCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAG
GGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGA
CTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAA
AAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGGCTTTTGCTGGCCTT
TTGCTCACATGTTCT (SEQ ID NO: 14)

FIGURE 7 (continued)

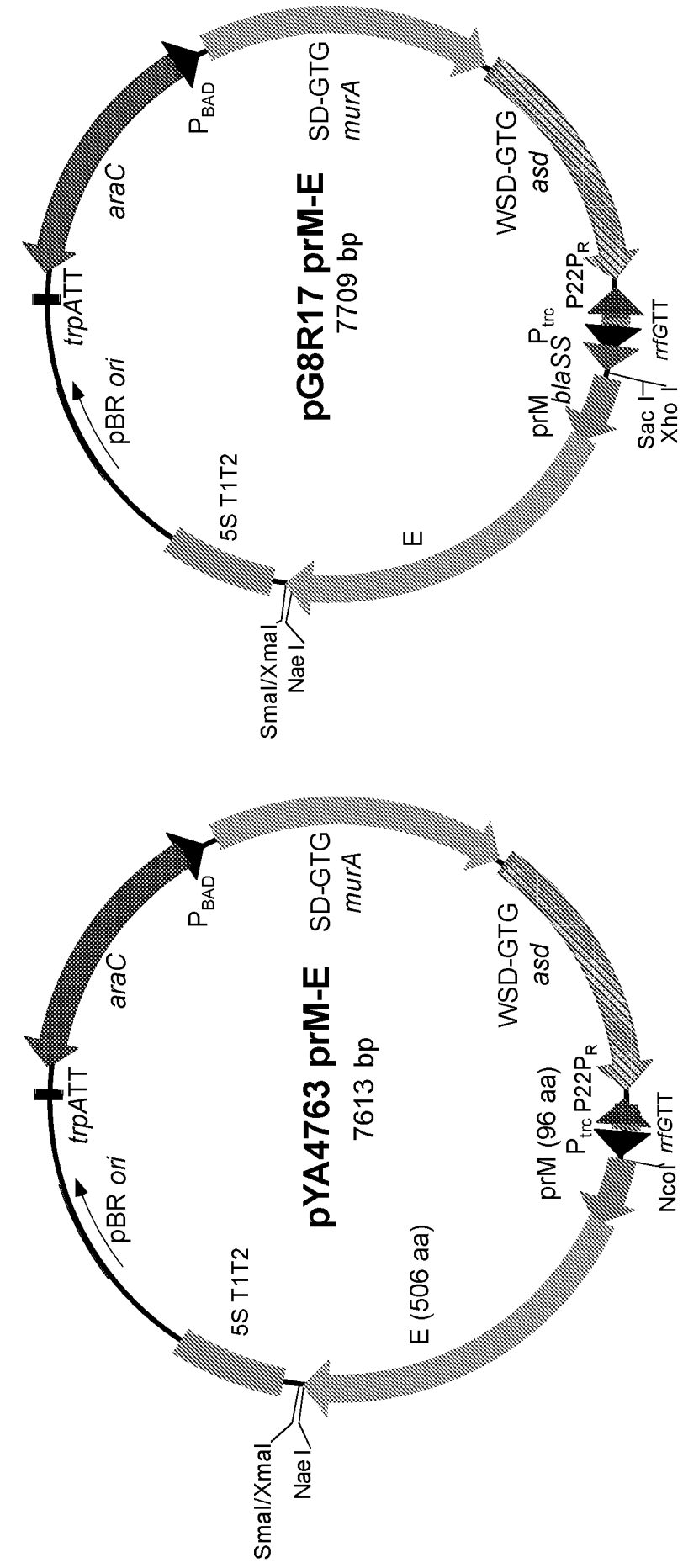
pG8R17 prM-E
7709 bp
pYA4763 prM-E
7613 bp
E (506 aa)
prM (96 aa)
E
P$_{BAD}$
SD-GTG
murA
WSD-GTG
asd
P22P$_R$
P$_{trc}$
rfGTT
blaSS
prM
Sac I
Xho I
NcoI
SmaI/XmaI
Nae I
5S T1T2
pBR ori
trpATT
araC

FIGURE 8

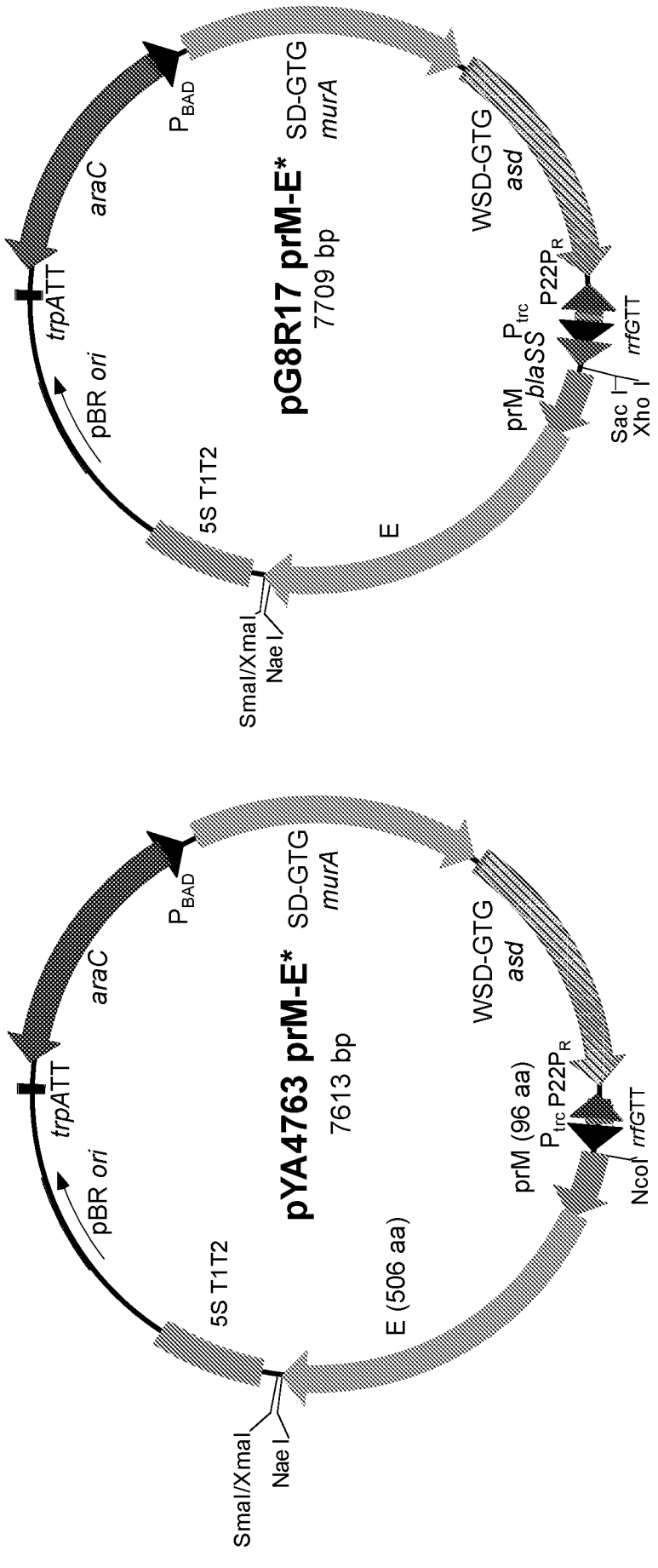
pG8R17 prM-E*
7709 bp
P_BAD
SD-GTG
murA
WSD-GTG
asd
P22P_R
P_trc
blaSS
prM
E
5S T1T2
pBR ori
trpATT
araC
SmaI/XmaI
Nae I
Sac I
Xho I
rrfGTT
pYA4763 prM-E*
7613 bp
E (506 aa)
prM (96 aa)
NcoI

FIGURE 9

prM 288 bp (96 aa)

GC ori 48.0& codon op 52.1% best codon 51.0%

```
1/1                                                 31/11
ATG AAA AAG GCG GAG GTC ACT AGA CGT GGG AGT GCA TAC TAT ATG TAC TTG GAC AGA AAC
ATG AAA AAG GCG GAG GTC ACT CGT CGT GGT AGC GCA TAC TAT ATG TAC CTG GAC CGC AAC
ATG AAA AAA GCG GAA GTT ACC CGT CGT GGT TCT GCG TAC TAC ATG TAC CTG GAT CGC AAC
M   K   K   A   E   V   T   R   R   G   S   A   Y   Y   M   Y   L   D   R   N

61/21                                               91/31
GAT GCT GGG GAG GCC ATA TCT TTT CCA ACC ACA TTG GGG ATG AAT AAG TGT TAT ATA CAG
GAT GCT GGC GAG GCC ATC TCT TTT CCA ACC ACC TTG GGT ATG AAT AAG TGT TAT ATC CAG
GAT GCG GGT GAA GCG ATC TCT TTC CCG ACC ACC CTG GGT ATG AAC AAA TGC TAC ATC CAG
D   A   G   E   A   I   S   F   P   T   T   L   G   M   N   K   C   Y   I   Q

121/41                                              151/51
ATC ATG GAT CTT GGA CAC ATG TGT GAT GCC ACC ATG AGC TAT GAA TGC CCT ATG CTG GAT
ATC ATG GAT CTG GGC CAC ATG TGT GAT GCC ACC ATG AGC TAT GAA TGC CCT ATG CTG GAT
ATC ATG GAT CTG GGT CAC ATG TGC GAT GCC ACC ATG TCT TAC GAA TGC CCG ATG CTG GAT
I   M   D   L   G   H   M   C   D   A   T   M   S   Y   E   C   P   M   L   D

181/61                                              211/71
GAG GGG GTG GAA CCA GAT GAC GTC GAT TGT TGG TGC AAC ACG ACG TCA ACT TGG GTT GTG
GAG GGG GTG GAA CCA GAT GAC GTC GAT TGT TGG TGC AAC ACG ACG TCA ACT TGG GTT GTG
GAA GGT GTT GAA CCG GAT GAT GTT GAT TGC TGG TGC AAC ACC ACC TCT ACT TGG GTT GTT
E   G   V   E   P   D   D   V   D   C   W   C   N   T   T   S   T   W   V   V

241/81                                              271/91
TAC GGA ACC TGC CAT CAC AAA AAA GGT GAA GCA CGG AGA TCT AGA AGA (SEQ ID NO: 15)
TAC GGT ACC TGC CAT CAC AAA AAA GGT GAA GCA CGC CGC TCT CGC CGC (SEQ ID NO: 16)
TAC GGT ACC TGC CAC CAC AAA AAA GGT GAA GCG CGT CGT TCT CGT CGT (SEQ ID NO: 17)
Y   G   T   C   H   H   K   K   G   E   A   R   R   S   R   R   (SEQ ID NO: 18)
```

FIGURE 10A

E protein (M) 1518 bp (506 aa)

GC ori 49.6% codon opt 52.1% best codon 52.0%

```
1/1                                                 31/11
ATG AAA ATC AGG TGC ATA GGA GTC AGC AAT AGG GAC TTT GTG GAA GGT ATG TCA GGT GGG
ATG AAA ATC CGC TGC ATC GGT GTC AGC AAT CGT GAC TTT GTG GAA GGT ATG TCC GGT GGT
ATG AAA ATC CGC TGC ATC GGT GTT TCT AAC CGT GAC TTC GTT GAA GGT ATG TCT GGT GGT
M   K   I   R   C   I   G   V   S   N   R   D   F   V   E   G   M   S   G   G

61/21                                               91/31
ACT TGG GTT GAT GTT GTC TTG GAA CAT GGA GGT TGT GTC ACC GTA ATG GCA CAG GAC AAA
ACT TGG GTT GAT GTT GTC CTG GAA CAT GGC GGT TGT GTC ACC GTA ATG GCA CAG GAC AAA
ACC TGG GTT GAT GTT GTT CTG GAA CAC GGT GGT TGC GTT ACC GTT ATG GCG CAG GAT AAA
T   W   V   D   V   V   L   E   H   G   G   C   V   T   V   M   A   Q   D   K

121/41                                              151/51
CCG ACT GTC GAC ATA GAG CTG GTT ACA ACA ACA GTC AGC AAC ATG GCG GAG GTA AGA TCC
CCG ACT GTC GAC ATC GAG CTG GTT ACC ACC ACC GTC AGC AAC ATG GCG GAG GTA CGT TCC
CCG ACC GTT GAT ATC GAA CTG GTT ACC ACC ACC GTT TCT AAC ATG GCG GAA GTT CGT TCT
P   T   V   D   I   E   L   V   T   T   T   V   S   N   M   A   E   V   R   S


181/61                                              211/71
TAC TGC TAT GAG GCA TCA ATA TCA GAC ATG GCT TCT GAC AGC CGC TGC CCA ACA CAA GGT
TAC TGC TAT GAG GCA TCC ATC TCC GAC ATG GCT TCT GAC AGC CGC TGC CCA ACC CAA GGT
TAC TGC TAC GAA GCG TCT ATC TCT GAT ATG GCG TCT GAT AGC CGT TGC CCG ACC CAG GGT
Y   C   Y   E   A   S   I   S   D   M   A   S   D   S   R   C   P   T   Q   G

241/81                                              271/91
GAA GCC TAC CTT GAC AAG CAA TCA GAC ACT CAA TAT GTC TGC AAA AGA ACG TTA GTG GAC
GAA GCC TAC CTT GAC AAG CAA TCC GAC ACT CAA TAT GTC TGC AAA CGT ACG CTG GTG GAC
GAA GCG TAC CTG GAT AAA CAG TCT GAT ACC CAG TAC GTT TGC AAA CGT ACC CTG GTT GAT
E   A   Y   L   D   K   Q   S   D   T   Q   Y   V   C   K   R   T   L   V   D

301/101                                             331/111
AGA GGC TGG GGA AAT GGA TGT GGA CTT TTT GGC AAA GGG AGC CTG GTG ACA TGC GCT AAG
CGT GGC TGG GGC AAT GGT TGT GGC CTT TTT GGT AAA GGG AGC CTG GTG ACC TGC GCT AAG
CGT GGT TGG GGC AAC GGT TGC GGT CTG TTC GGT AAA GGT TCT CTG GTT ACC TGC GCT AAA
R   G   W   G   N   G   C   G   L   F   G   K   G   S   L   V   T   C   A   K

361/121                                             391/131
TTT GCA TGC TCC AAG AAA ATG ACC GGG AAG AGC ATC CAG CCA GAG AAT CTG GAG TAC CGG
TTT GCA TGC TCC AAG AAA ATG ACC GGC AAG AGC ATC CAG CCA GAG AAT CTG GAG TAC CGC
TTC GCA TGC TCT AAA AAA ATG ACC GGT AAA AGC ATC CAG CCG GAA AAC CTG GAA TAC CGT
F   A   C   S   K   K   M   T   G   K   S   I   Q   P   E   N   L   E   Y   R
```

FIGURE 10B

```
421/141                                                 451/151
ATA ATG CTG TCA GTT CAT GGC TCC CAG CAC AGT GGG ATG ATC GTT AAT GAC ACA GGA CAT
ATC ATG CTG TCA GTT CAT GGC TCC CAG CAC AGT GGG ATG ATC GTT CAG GAC ACA GGT CAT
ATC ATG CTG TCT GTT CAC GGC TCC CAG CAC TCT GGT ATG ATC GTT AAC GAT ACC GGT CAC
I   M   L   S   V   H   G   S   Q   H   S   G   M   I   V   N   D   T   G   H
                                                                Q*

481/161                                                 511/171
GAA ACT GAT GAG AAT AGA GCG AAA GTT GAG ATA ACG CCC AAT TCA CCG AGA GCC GAA GCC
GAA ACT GAT GAG AAT CGC GCG AAA GTT GAG ATC ACG CCC AAT TCA CCG CGT GCC GAA GCC
GAA ACC GAT GAA AAC CGT GCG AAA GTT GAA ATC ACC CCG AAC TCT CCG CGT GCC GAA GCC
E   T   D   E   N   R   A   K   V   E   I   T   P   N   S   P   R   A   E   A

541/181                                                 571/191
ACC CTG GGG GGT TTT GGA AGC CTA GGA CTT GAT TGT GAA CCG AGG ACA GGC CTT GAC TTT
ACC CTG GGG GGT TTT GGT AGC CTA GGT CTT GAT TGT GAA CCG CGT ACA GGC CTT GAC TTT
ACC CTG GGT GGT TTC GGT TCT CTG GGT CTG GAT TGC GAA CCG CGT ACC GGT CTG GAT TTC
T   L   G   G   F   G   S   L   G   L   D   C   E   P   R   T   G   L   D   F

601/201                                                 631/211
TCA GAT TTG TAT TAC TTG ACT ATG AAT AAC AAG CAC TGG TTG GTT CAC AAG GAG TGG TTC
TCA GAT TTG TAT TAC TTG ACT ATG AAT AAC AAG CAC TGG TTG GTT CAC AAG GAG TGG TTC
TCT GAT CTG TAC TAC CTG ACC ATG AAC AAC AAG CAC TGG CTG GTT CAC AAG GAA TGG TTC
S   D   L   Y   Y   L   T   M   N   N   K   H   W   L   V   H   K   E   W   F

661/221                                                 691/231
CAC GAC ATT CCA TTA CCT TGG CAC GCT GGG GCA GAC ACC GGA ACT CCA CAC TGG AAC AAC
CAC GAC ATT CCA TTA CCT TGG CAC GCT GGG GCA GAC ACC GGT ACT CCA CAC TGG AAC AAC
CAC GAT ATC CCG CTG CCG TGG CAC GCT GGT GCA GAT ACC GGT ACC CCG CAC TGG AAC AAC
H   D   I   P   L   P   W   H   A   G   A   D   T   G   T   P   H   W   N   N

721/241                                                 751/251
AAA GAA GCA CTG GTA GAG TTC AAG GAC GCA CAT GCC AAA AGG CAA ACT GTC GTG GTT CTA
AAA GAA GCA CTG GTA GAG TTC AAG GAC GCA CAT GCC AAA CGT CAA ACT GTC GTG GTT CTG
AAA GAA GCA CTG GTT GAA TTC AAA GAT GCA CAC GCC AAA CGT CAG ACC GTT GTT GTT CTG
K   E   A   L   V   E   F   K   D   A   H   A   K   R   Q   T   V   V   V   L

781/261                                                 811/271
GGG AGT CAA GAA GGA GCA GTT CAC ACG GCC CTT GCT GGA GCT CTG GAG GCT GAG ATG GAT
GGG AGT CAA GAA GGT GCA GTT CAC ACG GCC CTT GCT GGT GCT CTG GAG GCT GAG ATG GAT
GGT TCT CAG GAA GGT GCA GTT CAC ACC GCC CTG GCT GGT GCT CTG GAA GCT GAA ATG GAT
G   S   Q   E   G   A   V   H   T   A   L   A   G   A   L   E   A   E   M   D

841/281                                                 871/291
GGT GCA AAG GGA AGG CTG TCC TCT GGC CAC TTG AAA TGT CGC CTG AAA ATG GAT AAA CTT
GGT GCA AAG GGC CGC CTG TCC TCT GGC CAC TTG AAA TGT CGC CTG AAA ATG GAT AAA CTT
GGT GCA AAA GGT CGT CTG TCT TCT GGT CAC CTG AAA TGC CGT CTG AAA ATG GAT AAA CTG
G   A   K   G   R   L   S   S   G   H   L   K   C   R   L   K   M   D   K   L
```

FIGURE 10B (continued)

```
901/301                                                 931/311
AGA TTG AAG GGC GTG TCA TAC TCC TTG TGT ACT GCA GCG TTC ACA TTC ACC AAG ATC CCG
CGT TTG AAG GGT GTG TCA TAC TCC TTG TGT ACT GCA GCG TTC ACA TTC ACC AAG ATC CCG
CGT CTG AAA GGT GTT TCT TAC TCT CTG TGC ACC GCA GCG TTC ACC TTC ACC AAA ATC CCG
R   L   K   G   V   S   Y   S   L   C   T   A   A   F   T   F   T   K   I   P

961/321                                                 991/331
GCT GAA ACA CTG CAC GGG ACA GTC ACA GTG GAG TTA CAG TAC GCA GGG ACA GAT GGA CCT
GCT GAA ACA CTG CAC GGG ACA GTC ACA GTG GAG TTA CAG TAC GCA GGG ACA GAT GGT CCT
GCT GAA ACC CTG CAC GGT ACC GTT ACC GTG GAA CTG CAG TAC GCA GGT ACC GAT GGT CCG
A   E   T   L   H   G   T   V   T   V   E   L   Q   Y   A   G   T   D   G   P

1021/341                                                1051/351
TGC AAG GTT CCA GCT CAG ATG GCG GTG GAC ATG CAA ACT CTG ACC CCA GTT GGG AGG TTG
TGC AAG GTT CCA GCT CAG ATG GCG GTG GAC ATG CAA ACT CTG ACC CCA GTT GGG CGT TTG
TGC AAA GTT CCG GCT CAG ATG GCG GTG GAT ATG CAG ACC CTG ACC CCG GTT GGT CGT CTG
C   K   V   P   A   Q   M   A   V   D   M   Q   T   L   T   P   V   G   R   L

1081/361                                                1111/371
ATA ACC GCT AAC CCC GTA ATC ACT GAA AGC ACT GAG AAC TCT AAG ATG ATG CTG GAA CTT
ATC ACC GCT AAC CCG GTA ATC ACT GAA AGC ACT GAG AAC TCT AAG ATG ATG CTG GAA CTT
ATC ACC GCT AAC CCG GTT ATC ACC GAA TCT ACC GAA AAC TCT AAA ATG ATG CTG GAA CTG
I   T   A   N   P   V   I   T   E   S   T   E   N   S   K   M   M   L   E   L

1141/381                                                1171/391
GAT CCA CCA TTT GGG GAC TCT TAC ATT GTC ATA GGA GTC GGG GAG AAG AAG ATC ACC CAC
GAT CCA CCA TTT GGG GAC TCT TAC ATT GTC ATC GGT GTC GGG GAG AAG AAG ATC ACC CAC
GAT CCG CCA TTC GGT GAT TCT TAC ATC GTT ATC GGT GTG GGT GAA AAA AAA ATC ACC CAC
D   P   P   F   G   D   S   Y   I   V   I   G   V   G   E   K   K   I   T   H

1201/401                                                1231/411
CAC TGG CAC AGG AGT GGC AGC ACC ATT GGA AAA GCA TTT GAA GCC ACT GTG AGA GGT GCC
CAC TGG CAC CGC AGT GGC AGC ACC ATT GGT AAA GCA TTT GAA GCC ACT GTG CGT GGT GCC
AAA CGT ATG GCA GTT CTG GGT GAT ACA GCC TGG GAT TTC GGT TCT GTT GGT GGT GCT CTG
H   W   H   R   S   G   S   T   I   G   K   A   F   E   A   T   V   R   G   A

1261/421                                                1291/431
AAG AGA ATG GCA GTC TTG GGA GAC ACA GCC TGG GAC TTT GGA TCA GTT GGA GGC GCT CTC
AAG CGT ATG GCA GTC TTG GGT GAC ACA GCC TGG GAC TTT GGT TCA GTT GGC GGC GCT CTC
AAA CGT ATG GCA GTT CTG GGT GAT ACA GCC TGG GAT TTC GGT TCT GTT GGT GGT GCT CTG
K   R   M   A   V   L   G   D   T   A   W   D   F   G   S   V   G   G   A   L

1321/441                                                1351/451
AAC TCA TTG GGC AAG GGC ATC CAT CAA ATT TTT GGA GCA GCT TTC AAA TCA TTG TTT GGA
AAC TCA TTG GGC AAG GGC ATC CAT CAA ATT TTT GGC GCA GCT TTC AAA TCA TTG TTT GGT
AAC TCT CTG GGT AAG GGT ATC CAC CAG ATC TTC GGT GCA GCT TTC AAA TCT CTG TTC GGT
N   S   L   G   K   G   I   H   Q   I   F   G   A   A   F   K   S   L   F   G
```

FIGURE 10B (continued)

```
1381/461                                                1411/471
GGA ATG TCC TGG TTC TCA CAA ATT CTC ATT GGA ACG TTG CTG ATG TGG TTG GGT CTG AAC
GGT ATG TCC TGG TTC TCA CAA ATT CTC ATT GGA ACG TTG CTG ATG TGG TTG GGT CTG AAC
GGT ATG TCT TGG TTC TCT CAA ATC CTG ATC GGT ACG CTG CTG ATG TGG CTG GGT CTG AAC
G   M   S   W   F   S   Q   I   L   I   G   T   L   L   M   W   L   G   L   N

1441/481                                                1471/491
ACA AAG AAT GGA TCT ATT TCC CTT ATG TGC TTG GCC TTA GGG GGA GTG TTG ATC TTC TTA
ACA AAG GAG GGC TCT ATT TCC CTT ATG TGC TTG GCC TTA GGG GGT GTG TTG ATC TTC TTA
ACC AAA AAC GGT TCT ATC TCT CTG ATG TGC CTG GCC CTG GGT GGT GTT CTG ATC TTC CTG
T   K   N   G   S   I   S   L   M   C   L   A   L   G   G   V   L   I   F   L
        E*  (SEQ ID NO: 23)
1501/501
TCC ACA GCC GTC TCT GCT (SEQ ID NO: 19)
TCC ACA GCC GTC TCT GCT (SEQ ID NO: 20)
TCT ACC GCC GTT TCT GCT (SEQ ID NO: 21)
S   T   A   V   S   A   (SEQ ID NO: 22)
```

FIGURE 10B (continued)

NS4A 381 bp (127aa) orig codon 53.8% GC codon opt 52.8% GC

Extra sequence added for 15 bp for Starting codon + 2 extra AAs at 5' and 2 AAs at 3' end) and NcoI-XmaI ends
Cut with NcoI and XmaI sites in pYA4763 (pBR *ori*), pYA4589 (p15A *ori*) and pYA4595 (pSC101 *ori*) lysis vectors.

```
1/1                                                 31/11
ATG GAA AAA GGA GCG GCT TTT GGA GTG ATG GAA GCC CTG GGA ACA CTG CCA GGA CAC ATG
   GGT GCT GCT TTC GGT GTT ATG GAA GCT CTG GGT ACC CTG CCG GGT CAC ATG
M  E   K   G   A   A   F   G   V   M   E   A   L   G   T   L   P   G   H   M
          → NS4A
61/21                                               91/31
ACA GAG AGA TTC CAG GAA GCC ATT GAC AAC CTC GCT GTG CTC ATG CGG GCA GAG ACT GGA
ACC GAA CGT TTC CAG GAA GCT ATC GAT AAC CTG GCT GTT CTG ATG CGT GCT GAA ACC GGT
T   E   R   F   Q   E   A   I   D   N   L   A   V   L   M   R   A   E   T   G

121/41                                              151/51
AGC AGG CCT TAC AAA GCC GCG GCG GCC CAA TTG CCG GAG ACC CTA GAG ACC ATT ATG CTT
TCT CGT CCG TAC AAA GCT GCT GCT GCT CAG CTG CCG GAA ACC CTG GAA ACC ATC ATG CTG
S   R   P   Y   K   A   A   A   A   Q   L   P   E   T   L   E   T   I   M   L

181/61                                              211/71
TTG GGG TTG CTG GGA ACA GTC TCG CTG GGA ATC TTT TTC GTC TTG ATG AGG AAC AAG GGC
CTG GGT CTG CTG GGT ACC GTT TCT CTG GGT ATC TTC TTC GTT CTG ATG CGT AAC AAA GGT
L   G   L   L   G   T   V   S   L   G   I   F   F   V   L   M   R   N   K   G

241/81                                              271/91
ATA GGG AAG ATG GGC TTT GGA ATG GTG ACT CTT GGG GCC AGC GCA TGG CTC ATG TGG CTC
ATC GGT AAA ATG GGT TTC GGT ATG GTT ACC CTG GGT GCT TCT GCT TGG CTG ATG TGG CTG
I   G   K   M   G   F   G   M   V   T   L   G   A   S   A   W   L   M   W   L

301/101                                             331/111
TCG GAA ATT GAG CCA GCC AGA ATT GCA TGT GTC CTC ATT GTT GTG TTC CTA TTG CTG GTG
TCT GAA ATC GAA CCG GCT CGT ATC GCT TGT GTT CTG ATC GTT GTT TTC CTG CTG CTG GTT
S   E   I   E   P   A   R   I   A   C   V   L   I   V   V   F   L   L   L   V

361/121
GTG CTC ATA CCT GAG CCA GAA AAG CAA AGA (SEQ ID NO: 24)    original codon
GTT CTG ATC CCG GAA CCG GAA AAA CAC CGT GCC GGC TAATCCCGGG (SEQ ID NO: 25)
                                                          optimized codon
V   L   I   P   E   P   E   K   Q   R   A   G        XmaI  (SEQ ID NO: 26)
```

FIGURE 11A

Codon-optimized sequence with 6 x His tagged sequence

```
CC ATG GAA AAA GGT GCT GCT TTC GGT GTT ATG GAA GCT CTG GGT ACC CTG CCG GGT CAC
   M   E   K   G   A   A   F   G   V   M   E   A   L   G   T   L   P   G   H
ATG ACC GAA CGT TTC CAG GAA GCT ATC GAT AAC CTG GCT GTT CTG ATG CGT GCT GAA ACC
M   T   E   R   F   Q   E   A   I   D   N   L   A   V   L   M   R   A   E   T
GGT TCT CGT CCG TAC AAA GCT GCT GCT GCT CAG CTG CCG GAA ACC CTG GAA ACC ATC ATG
G   S   R   P   Y   K   A   A   A   A   Q   L   P   E   T   L   E   T   I   M
CTG CTG GGT CTG CTG GGT ACC GTT TCT CTG GGT ATC TTC TTC GTT CTG ATG CGT AAC AAA
L   L   G   L   L   G   T   V   S   L   G   I   F   F   V   L   M   R   N   K
GGT ATC GGT AAA ATG GGT TTC GGT ATG GTT ACC CTG GGT GCT TCT GCT TGG CTG ATG TGG
G   I   G   K   M   G   F   G   M   V   T   L   G   A   S   A   W   L   M   W
CTG TCT GAA ATC GAA CCG GCT CGT ATC GCT TGT GTT CTG ATC GTT GTT TTC CTG CTG CTG
L   S   E   I   E   P   A   R   I   A   C   V   L   I   V   V   F   L   L   L
                                                NaeI
GTT GTT CTG ATC CCG GAA CCG GAA AAA CAC CGT GCC GGC CAC CAT CAC CAT CAC CAT TAG
V   V   L   I   P   E   P   E   K   Q   R   A   G   H   H   H   H   H   H   *
 NaeI       XmaI
CCGGCTAATcccggg  (SEQ ID NO: 27)
                 (SEQ ID NO: 28, protein sequence)
```

FIGURE 11B

NS4B codon opt NcoI-SmaI 765 bp
Orig 52.5% GC best codon 52.7% GC 254 aa

Extra sequence added for 12 bp for Starting codon + 1 extra AA at 5' and 2 AAs at 3' end) and NcoI-XmaI ends

Cut with NcoI and XmaI sites in pYA4763 (pBR *ori*), pYA4589 (p15A *ori*) and pYA4595(pSC101 *ori*) lysis vectors.

```
1/1                                                 31/11
ATG GAA AAT GAA CTC GGA TGG TTG GAG AGA ACA AAG AGT GAC CTA AGC CAT CTA ATG GGA
    AAC GAA CTG GGT TGG CTG GAA CGT ACC AAA TCT GAT CTG TCT CAC CTG ATG GGT
M   E   N   E   L   G   W   L   E   R   T   K   S   D   L   S   H   L   M   G
        → NS4B
61/21                                               91/31
AGG AGA GAG GAG GGG GCA ACC ATG GGA TTC TCA ATG GAC ATT GAC CTG CGG CCA GCC TCA
CGT CGT GAA GAA GGT GCT ACC ATG GGT TTC TCT ATG GAT ATC GAT CTG CGT CCG GCT TCT
R   R   E   E   G   A   T   M   G   F   S   M   D   I   D   L   R   P   A   S

121/41                                              151/51
GCT TGG GCC ATC TAT GCT GCC TTG ACA ACT TTC ATT ACC CCA GCC GTC CAA CAT GCA GTG
GCT TGG GCT ATC TAC GCT GCT CTG ACC ACC TTC ATC ACC CCG GCT GTT CAG CAC GCT GTT
A   W   A   I   Y   A   A   L   T   T   F   I   T   P   A   V   Q   H   A   V

181/61                                              211/71
ACC ACT TCA TAC AAC AAC TAC TCC TTA ATG GCG ATG GCC ACG CAA GCT GGA GTG TTG TTT
ACC ACC TCT TAC AAC AAC TAC TCT CTG ATG GCT ATG GCT ACC CAG GCT GGT GTT CTG TTC
T   T   S   Y   N   N   Y   S   L   M   A   M   A   T   Q   A   G   V   L   F

241/81                                              271/91
GGT ATG GGC AAA GGG ATG CCA TTC TAC GCA TGG GAC TTT GGA GTC CCG CTG CTA ATG ATA
GGT ATG GGT AAA GGT ATG CCG TTC TAC GCG TGG GAT TTC GGT GTT CCG CTG CTG ATG ATC
G   M   G   K   G   M   P   F   Y   A   W   D   F   G   V   P   L   L   M   I

301/101                                             331/111
GGT TGC TAC TCA CAA TTA ACG CCC CTG ACC CTA ATA GTG GCC ATC ATT TTG CTC GTG GCG
GGT TGC TAC TCT CAG CTG ACC CCG CTG ACC CTG ATC GTT GCT ATC ATC CTG CTG GTT GCT
G   C   Y   S   Q   L   T   P   L   T   L   I   V   A   I   I   L   L   V   A

361/121                                             391/131
CAC TAC ATG TAC TTG ATC CCA GGG CTG CAG GCA GCA GCT GCG CGT GCT GCC CAG AAG AGA
CAC TAC ATG TAC CTG ATC CCG GGT CTG CAG GCT GCT GCT GCT CGT GCT GCT CAG AAA CGT
H   Y   M   Y   L   I   P   G   L   Q   A   A   A   A   R   A   A   Q   K   R

421/141                                             451/151
ACG GCA GCT GGC ATC ATG AAG AAC CCT GTT GTG GAT GGA ATA GTG GTG ACT GAC ATT GAC
ACC GCT GCT GGT ATC ATG AAA AAC CCG GTT GTT GAT GGT ATC GTT GTT ACC GAT ATC GAT
T   A   A   G   I   M   K   N   P   V   V   D   G   I   V   V   T   D   I   D
```

FIGURE 12A

```
481/161                                                 511/171
ACA ATG ACA ATT GAC CCC CAA GTG GAG AAA AAG ATG GGA CAG GTG CTA CTC ATG GCA GTA
ACC ATG ACC ATC GAT CCG CAG GTT GAA AAA AAA ATG GGT CAG GTT CTG CTG ATG GCT GTT
T   M   T   I   D   P   Q   V   E   K   K   M   G   Q   V   L   L   M   A   V

541/181                                                 571/191
GCC GTC TCC AGC GCC ATA CTG TCG CGG ACC GCC TGG GGG TGG GGG GAG GCT GGG GCC CTG
GCT GTT TCT TCT GCT ATC CTG TCT CGT ACC GCT TGG GGT TGG GGT GAA GCT GGT GCT CTG
A   V   S   S   A   I   L   S   R   T   A   W   G   W   G   E   A   G   A   L

601/201                                                 631/211
ATC ACA GCC GCA ACT TCC ACT TTG TGG GAA GGC TCT CCG AAC AAG TAC TGG AAC TCC TCT
ATC ACC GCT GCT ACC TCT ACC CTG TGG GAA GGT TCT CCG AAC AAA TAC TGG AAC TCT TCT
I   T   A   A   T   S   T   L   W   E   G   S   P   N   K   Y   W   N   S   S

661/221                                                 691/231
ACA GCC ACT TCA CTG TGT AAC ATT TTT AGG GGA AGT TAC TTG GCT GGA GCT TCT CTA ATC
ACC GCT ACC TCT CTG TGC AAC ATC TTC CGT GGT TCT TAC CTG GCT GGT GCT TCT CTG ATC
T   A   T   S   L   C   N   I   F   R   G   S   Y   L   A   G   A   S   L   I

721/241
TAC ACA GTA ACA AGA AAC GCT GGC TTG GTC AAG AGA  (SEQ ID NO: 29)    orig codon
TAC ACC GTT ACC CGT AAC GCT GGT CTG GTT AAA CGT GCC GGC TAA TCCCGGG (SEQ ID NO:
                                                                    30) opt codon
Y   T   V   T   R   N   A   G   L   V   K   R   A   G   *  XmaI (SEQ ID NO: 31)
```

FIGURE 12A (continued)

6 x His tagged sequence

```
   NcoI
CC ATG GAA AAC GAA CTG GGT TGG CTG GAA CGT ACC AAA TCT GAT CTG TCT CAC CTG ATG
   M   E   N   E   L   G   W   L   E   R   T   K   S   D   L   S   H   L   M
GGT CGT CGT GAA GAA GGT GCT ACT ATG GGT TTC TCT ATG GAT ATC GAT CTG CGT CCG GCT
G   R   R   E   E   G   A   T   M   G   F   S   M   D   I   D   L   R   P   A
TCT GCT TGG GCT ATC TAC GCT GCT CTG ACC ACC TTC ATC ACC CCG GCT GTT CAG CAC GCT
S   A   W   A   I   Y   A   A   L   T   T   F   I   T   P   A   V   Q   H   A
GTT ACC ACC TCT TAC AAC AAC TAC TCT CTG ATG GCT ATG GCT ACC CAG GCT GGT GTT CTG
V   T   T   S   Y   N   N   Y   S   L   M   A   M   A   T   Q   A   G   V   L
TTC GGT ATG GGT AAA GGT ATG CCG TTC TAC GCG TGG GAT TTC GGT GTT CCG CTG CTG ATG
F   G   M   G   K   G   M   P   F   Y   A   W   D   F   G   V   P   L   L   M
ATC GGT TGC TAC TCT CAG CTG ACC CCG CTG ACC CTG ATC GTT GCT ATC ATC CTG CTG GTT
I   G   C   Y   S   Q   L   T   P   L   T   L   I   V   A   I   I   L   L   V
GCT CAC TAC ATG TAC CTG ATT CCG GGT CTG CAG GCT GCT GCT GCT CGT GCT GCT CAG AAA
A   H   Y   M   Y   L   I   P   G   L   Q   A   A   A   A   R   A   A   Q   K
```

FIGURE 12B

```
CGT ACC GCT GCT GGT ATC ATG AAA AAC CCG GTT GTT GAT GGT ATC GTT GTT ACC GAT ATC
R   T   A   A   G   I   M   K   N   P   V   V   D   G   I   V   V   T   D   I
GAT ACC ATG ACC ATC GAT CCG CAG GTT GAA AAA AAA ATG GGT CAG GTT CTG CTG ATG GCT
D   T   M   T   I   D   P   Q   V   E   K   K   M   G   Q   V   L   L   M   A
GTT GCT GTT TCT TCT GCT ATC CTG TCT CGT ACC GCT TGG GGT TGG GGT GAA GCT GGT GCT
V   A   V   S   S   A   I   L   S   R   T   A   W   G   W   G   E   A   G   A
CTG ATC ACC GCT GCT ACC TCT ACC CTG TGG GAA GGT TCT CCG AAC AAA TAC TGG AAC TCT
L   I   T   A   A   T   S   T   L   W   E   G   S   P   N   K   Y   W   N   S
TCT ACC GCT ACC TCT CTG TGC AAC ATC TTC CGT GGT TCT TAC CTG GCT GGT GCT TCT CTG
S   T   A   T   S   L   C   N   I   F   R   G   S   Y   L   A   G   A   S   L
                                                    NaeI
ATC TAC ACC GTT ACC CGT AAC GCT GGT CTG GTT AAA CGT GCC GGC CAC CAT CAC CAT CAC
I   Y   T   V   T   R   N   A   G   L   V   K   R   A   G   H   H   H   H   H
        NaeI        XmaI
CAT TAG CCGGCTAATcccggg  (SEQ ID NO: 32)
H   *                (SEQ ID NO: 33)
```

FIGURE 12B (continued)

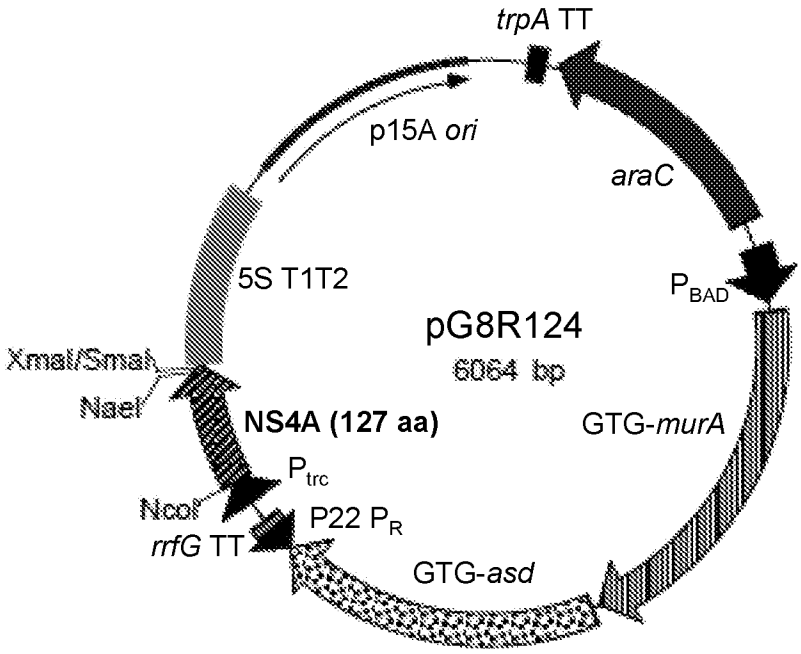

FIGURE 13

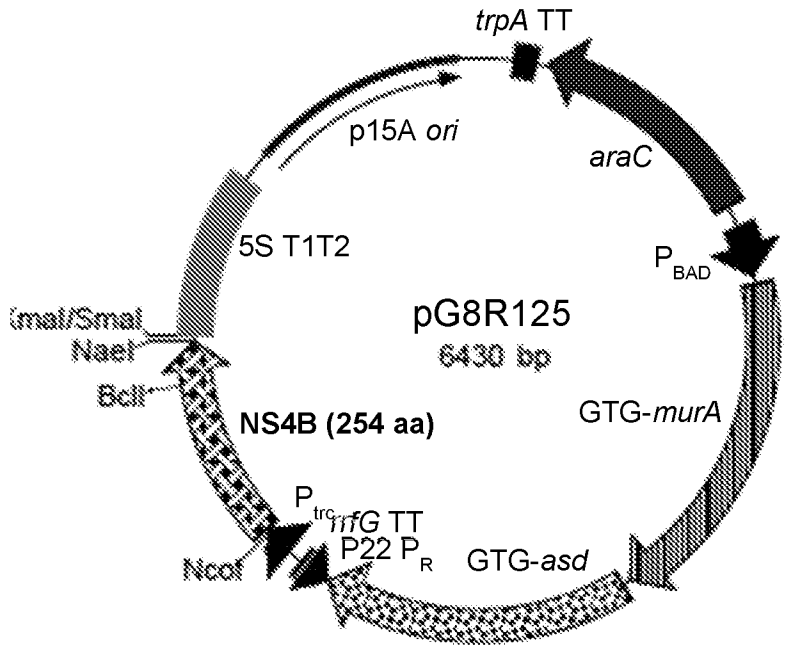

FIGURE 14

prM-E 1800 bp (600 aa)
original 48.0 GC & codon op 51.9% GC

Cut with NcoI and XmaI sites in pYA4763 (pBR *ori*), pYA4589 (p15A *ori*) and pYA4595 (pSC101 *ori*) lysis vector.

N-glycosylation sites (bolded, aa 254, and 577) are unchanged.

NcoI-XmaI ends tagged

```
1/1                                                 31/11
ATG GAA AAA GCG GAG GTC ACT AGA CGT GGG AGT GCA TAC TAT ATG TAC TTG GAC AGA AAC
ATG GAA AAA GCG GAA GTT ACC CGT CGT GGT TCT GCG TAC TAC ATG TAC CTG GAT CGC AAC
M   E   K   A   E   V   T   R   R   G   S   A   Y   Y   M   Y   L   D   R   N
          → prM protein
61/21                                               91/31
GAT GCT GGG GAG GCC ATA TCT TTT CCA ACC ACA TTG GGG ATG AAT AAG TGT TAT ATA CAG
GAT GCG GGT GAA GCG ATC TCT TTC CCG ACC ACC CTG GGT ATG AAC AAA TGC TAC ATC CAG
D   A   G   E   A   I   S   F   P   T   T   L   G   M   N   K   C   Y   I   Q
```

FIGURE 15A

```
121/41                                                151/51
ATC ATG GAT CTT GGA CAC ATG TGT GAT GCC ACC ATG AGC TAT GAA TGC CCT ATG CTG GAT
ATC ATG GAT CTG GGT CAC ATG TGC GAT GCC ACC ATG TCT TAC GAA TGC CCG ATG CTG GAT
I   M   D   L   G   H   M   C   D   A   T   M   S   Y   E   C   P   M   L   D

181/61                                                211/71
GAG GGG GTG GAA CCA GAT GAC GTC GAT TGT TGG TGC AAC ACG ACG TCA ACT TGG GTT GTG
GAA GGT GTT GAA CCG GAT GAT GTT GAT TGC TGG TGC AAC ACC ACC TCT ACT TGG GTT GTT
E   G   V   E   P   D   D   V   D   C   W   C   N   T   T   S   T   W   V   V

241/81                                                271/91
TAC GGA ACC TGC CAT CAC AAA AAA GGT GAA GCA CGG AGA TCT AGA AGA ATC AGG TGC ATA
TAC GGT ACC TGC CAC CAC AAA AAA GGT GAA GCG CGT CGT TCT CGT CGT ATC CGC TGC ATC
Y   G   T   C   H   H   K   K   G   E   A   R   R   S   R   R   I   R   C   I
                                                                  → E protein
301/101                                               331/111
GGA GTC AGC AAT AGG GAC TTT GTG GAA GGT ATG TCA GGT GGG ACT TGG GTT GAT GTT GTC
GGT GTT TCT AAC CGT GAC TTC GTT GAA GGT ATG TCT GGT GGT ACC TGG GTT GAT GTT GTT
G   V   S   N   R   D   F   V   E   G   M   S   G   G   T   W   V   D   V   V

361/121                                               391/131
TTG GAA CAT GGA GGT TGT GTC ACC GTA ATG GCA CAG GAC AAA CCG ACT GTC GAC ATA GAG
CTG GAA CAC GGT GGT TGC GTT ACC GTT ATG GCG CAG GAT AAA CCG ACC GTT GAT ATC GAA
L   E   H   G   G   C   V   T   V   M   A   Q   D   K   P   T   V   D   I   E

421/141                                               451/151
CTG GTT ACA ACA ACA GTC AGC AAC ATG GCG GAG GTA AGA TCC TAC TGC TAT GAG GCA TCA
CTG GTT ACC ACC ACC GTT TCT AAC ATG GCG GAA GTT CGT TCT TAC TGC TAC GAA GCG TCT
L   V   T   T   T   V   S   N   M   A   E   V   R   S   Y   C   Y   E   A   S

481/161                                               511/171
ATA TCA GAC ATG GCT TCT GAC AGC CGC TGC CCA ACA CAA GGT GAA GCC TAC CTT GAC AAG
ATC TCT GAT ATG GCG TCT GAT AGC CGT TGC CCG ACC CAG GGT GAA GCG TAC CTG GAT AAA
I   S   D   M   A   S   D   S   R   C   P   T   Q   G   E   A   Y   L   D   K

541/181                                               571/191
CAA TCA GAC ACT CAA TAT GTC TGC AAA AGA ACG TTA GTG GAC AGA GGC TGG GGA AAT GGA
CAG TCT GAT ACC CAG TAC GTT TGC AAA CGT ACC CTG GTT GAT CGT GGT TGG GGC AAC GGT
Q   S   D   T   Q   Y   V   C   K   R   T   L   V   D   R   G   W   G   N   G

601/201                                               631/211
TGT GGA CTT TTT GGC AAA GGG AGC CTG GTG ACA TGC GCT AAG TTT GCA TGC TCC AAG AAA
TGC GGT CTG TTC GGT AAA GGT TCT CTG GTT ACC TGC GCT AAA TTC GCA TGC TCT AAA AAA
C   G   L   F   G   K   G   S   L   V   T   C   A   K   F   A   C   S   K   K

661/221                                               691/231
ATG ACC GGG AAG AGC ATC CAG CCA GAG AAT CTG GAG TAC CGG ATA ATG CTG TCA GTT CAT
ATG ACC GGT AAA AGC ATC CAG CCG GAA AAC CTG GAA TAC CGT ATC ATG CTG TCT GTT CAC
M   T   G   K   S   I   Q   P   E   N   L   E   Y   R   I   M   L   S   V   H
```

FIGURE 15A (continued)

```
721/241                                                 751/251
GGC TCC CAG CAC AGT GGG ATG ATC GTT AAT GAC ACA GGA CAT GAA ACT GAT GAG AAT AGA
GGC TCC CAG CAC WCT GGT ATG ATC GTT AAC GAT ACC GGT CAC GAA ACC GAT GAA AAC CGT
G   S   Q   H   S   G   M   I   V   N   D   T   G   H   E   T   D   E   N   R

781/261                                                 811/271
GCG AAA GTT GAG ATA ACG CCC AAT TCA CCG AGA GCC GAA GCC ACC CTG GGG GGT TTT GGA
GCG AAA GTT GAA ATC ACC CCG AAC TCT CCG CGT GCC GAA GCC ACC CTG GGT GGT TTC GGT
A   K   V   E   I   T   P   N   S   P   R   A   E   A   T   L   G   G   F   G

841/281                                                 871/291
AGC CTA GGA CTT GAT TGT GAA CCG AGG ACA GGC CTT GAC TTT TCA GAT TTG TAT TAC TTG
TCT CTG GGT CTG GAT TGC GAA CCG CGT ACC GGT CTG GAT TTC TCT GAT CTG TAC TAC CTG
S   L   G   L   D   C   E   P   R   T   G   L   D   F   S   D   L   Y   Y   L

901/301                                                 931/311
ACT ATG AAT AAC AAG CAC TGG TTG GTT CAC AAG GAG TGG TTC CAC GAC ATT CCA TTA CCT
ACC ATG AAC AAC AAG CAC TGG CTG GTT CAC AAG GAA TGG TTC CAC GAT ATC CCG CTG CCG
T   M   N   N   K   H   W   L   V   H   K   E   W   F   H   D   I   P   L   P

961/321                                                 991/331
TGG CAC GCT GGG GCA GAC ACC GGA ACT CCA CAC TGG AAC AAC AAA GAA GCA CTG GTA GAG
TGG CAC GCT GGT GCA GAT ACC GGT ACC CCG CAC TGG AAC AAC AAA GAA GCA CTG GTT GAA
W   H   A   G   A   D   T   G   T   P   H   W   N   N   K   E   A   L   V   E

1021/341                                                1051/351
TTC AAG GAC GCA CAT GCC AAA AGG CAA ACT GTC GTG GTT CTA GGG AGT CAA GAA GGA GCA
TTC AAA GAT GCA CAC GCC AAA CGT CAG ACC GTT GTT GTT CTG GGT TCT CAG GAA GGT GCA
F   K   D   A   H   A   K   R   Q   T   V   V   V   L   G   S   Q   E   G   A

1081/361                                                1111/371
GTT CAC ACG GCC CTT GCT GGA GCT CTG GAG GCT GAG ATG GAT GGT GCA AAG GGA AGG CTG
GTT CAC ACC GCC CTG GCT GGT GCT CTG GAA GCT GAA ATG GAT GGT GCA AAA GGT CGT CTG
V   H   T   A   L   A   G   A   L   E   A   E   M   D   G   A   K   G   R   L

1141/381                                                1171/391
TCC TCT GGC CAC TTG AAA TGT CGC CTG AAA ATG GAT AAA CTT AGA TTG AAG GGC GTG TCA
TCT TCT GGT CAC CTG AAA TGC CGT CTG AAA ATG GAT AAA CTG CGT CTG AAA GGT GTT TCT
S   S   G   H   L   K   C   R   L   K   M   D   K   L   R   L   K   G   V   S

1201/401                                                1231/411
TAC TCC TTG TGT ACT GCA GCG TTC ACA TTC ACC AAG ATC CCG GCT GAA ACA CTG CAC GGG
TAC TCT CTG TGC ACC GCA GCG TTC ACC TTC ACC AAA ATC CCG GCT GAA ACC CTG CAC GGT
Y   S   L   C   T   A   A   F   T   F   T   K   I   P   A   E   T   L   H   G

1261/421                                                1291/431
ACA GTC ACA GTG GAG TTA CAG TAC GCA GGG ACA GAT GGA CCT TGC AAG GTT CCA GCT CAG
ACC GTT ACC GTG GAA CTG CAG TAC GCA GGT ACC GAT GGT CCG TGC AAA GTT CCG GCT CAG
T   V   T   V   E   L   Q   Y   A   G   T   D   G   P   C   K   V   P   A   Q
```

FIGURE 15A (continued)

```
1321/441                                                1351/451
ATG GCG GTG GAC ATG CAA ACT CTG ACC CCA GTT GGG AGG TTG ATA ACC GCT AAC CCC GTA
ATG GCG GTG GAY ATG CAR ACS CTG ACC CCS GTT GGY CGY CTG ATC ACC GCT AAC CCG GTY
M   A   V   D   M   Q   T   L   T   P   V   G   R   L   I   T   A   N   P   V

1381/461                                                1411/471
ATC ACT GAA AGC ACT GAG AAC TCT AAG ATG ATG CTG GAA CTT GAT CCA CCA TTT GGG GAC
ATC ACS GAA TCY ACS GAR AAC TCT AAR ATG ATG CTG GAA CTS GAT CCS CCS TTC GGY GAY
I   T   E   S   T   E   N   S   K   M   M   L   E   L   D   P   P   F   G   D

1441/481                                                1471/491
TCT TAC ATT GTC ATA GGA GTC GGG GAG AAG AAG ATC ACC CAC CAC TGG CAC AGG AGT GGC
TCT TAC ATC GTY ATC GGY GTS GGY GAR AAR AAR ATC ACC CAC CAC TGG CAC CGC TCT GGC
S   Y   I   V   I   G   V   G   E   K   K   I   T   H   H   W   H   R   S   G

1501/501                                                1531/511
AGC ACC ATT GGA AAA GCA TTT GAA GCC ACT GTG AGA GGT GCC AAG AGA ATG GCA GTC TTG
TCT ACC ATC GGY AAA GCA TTC GAA GCC ACS GTY CGY GGT GCC AAR CGY ATG GCA GTY CTG
S   T   I   G   K   A   F   E   A   T   V   R   G   A   K   R   M   A   V   L

1561/521                                                1591/531
GGA GAC ACA GCC TGG GAC TTT GGA TCA GTT GGA GGC GCT CTC AAC TCA TTG GGC AAG GGC
GGY GAY ACA GCC TGG GAY TTC GGY TCY GTT GGY GGY GCT CTS AAC TCY CTG GGY AAA GGY
G   D   T   A   W   D   F   G   S   V   G   G   A   L   N   S   L   G   K   G

1621/541                                                1651/551
ATC CAT CAA ATT TTT GGA GCA GCT TTC AAA TCA TTG TTT GGA GGA ATG TCC TGG TTC TCA
ATC CAC CAR ATC TTC GGY GCA GCT TTC AAA TCY CTG TTC GGY GGY ATG TCY TGG TTC TCY
I   H   Q   I   F   G   A   A   F   K   S   L   F   G   G   M   S   W   F   S

1681/561                                                1711/571
CAA ATT CTC ATT GGA ACG TTG CTG ATG TGG TTG GGT CTG AAC ACA AAG AAT GGA TCT ATT
CAA ATC CTS ATC GGY ACS CTG CTG ATG TGG CTG GGT CTG AAC ACS AAR AAC GGY TCT ATC
Q   I   L   I   G   T   L   L   M   W   L   G   L   N   T   K   N   G   S   I

1741/581                                                1771/591
TCC CTT ATG TGC TTG GCC TTA GGG GGA GTG TTG ATC TTC TTA TCC ACA GCC GTC TCT GCT
TCY CTS ATG TGC CTG GCC CTS GGY GGY GTY CTG ATC TTC CTS TCY ACS GCC GTY TCT GCT
S   L   M   C   L   A   L   G   G   V   L   I   F   L   S   T   A   V   S   A

1801/601
GCC GGC TAA TCC CGG G   (SEQ ID NO: 34)
                        (SEQ ID NO: 35)
A   G   *     XmaI      (SEQ ID NO: 36)
```

FIGURE 15A (continued)

6 x His tagged sequence

```
CC ATG GAA AAA GCG GAA GTT ACC CGT CGT GGT TCT GCG TAC TAC ATG TAC CTG GAT CGC
   M   E   K   A   E   V   T   R   R   G   S   A   Y   Y   M   Y   L   D   R
AAC GAT GCG GGT GAA GCG ATC TCT TTC CCG ACC ACC CTG GGT ATG AAC AAA TGC TAC ATC
N   D   A   G   E   A   I   S   F   P   T   T   L   G   M   N   K   C   Y   I
CAG ATC ATG GAT CTG GGT CAC ATG TGC GAT GCC ACC ATG TCT TAC GAA TGC CCG ATG CTG
Q   I   M   D   L   G   H   M   C   D   A   T   M   S   Y   E   C   P   M   L
GAT GAA GGT GTT GAA CCG GAT GAT GTT GAT TGC TGG TGC AAC ACC ACC TCT ACT TGG GTT
D   E   G   V   E   P   D   D   V   D   C   W   C   N   T   T   S   T   W   V
GTT TAC GGT ACC TGC CAC CAC AAA AAA GGT GAA GCG CGT CGT TCT CGT CGT ATC CGC TGC
V   Y   G   T   C   H   H   K   K   G   E   A   R   R   S   R   R   I   R   C
ATC GGT GTT TCT AAC CGT GAC TTC GTT GAA GGT ATG TCT GGT GGT ACC TGG GTT GAT GTT
I   G   V   S   N   R   D   F   V   E   G   M   S   G   G   T   W   V   D   V
GTT CTG GAA CAC GGT GGT TGC GTT ACC GTT ATG GCG CAG GAT AAA CCG ACC GTT GAT ATC
V   L   E   H   G   G   C   V   T   V   M   A   Q   D   K   P   T   V   D   I
GAA CTG GTT ACC ACC ACC GTT TCT AAC ATG GCG GAA GTT CGT TCT TAC TGC TAC GAA GCG
E   L   V   T   T   T   V   S   N   M   A   E   V   R   S   Y   C   Y   E   A
TCT ATC TCT GAT ATG GCG TCT GAT AGC CGT TGC CCG ACC CAG GGT GAA GCG TAC CTG GAT
S   I   S   D   M   A   S   D   S   R   C   P   T   Q   G   E   A   Y   L   D
AAA CAG TCT GAT ACC CAG TAC GTT TGC AAA CGT ACC CTG GTT GAT CGT GGT TGG GGC AAC
K   Q   S   D   T   Q   Y   V   C   K   R   T   L   V   D   R   G   W   G   N
GGT TGC GGT CTG TTC GGT AAA GGT TCT CTG GTT ACC TGC GCT AAA TTC GCA TGC TCT AAA
G   C   G   L   F   G   K   G   S   L   V   T   C   A   K   F   A   C   S   K
AAA ATG ACC GGT AAA AGC ATC CAG CCG GAA AAC CTG GAA TAC CGT ATC ATG CTG TCT GTT
K   M   T   G   K   S   I   Q   P   E   N   L   E   Y   R   I   M   L   S   V
CAC GGC TCC CAG CAC TCT GGT ATG ATC GTT AAC GAT ACC GGT CAC GAA ACC GAT GAA AAC
H   G   S   Q   H   S   G   M   I   V   N   D   T   G   H   E   T   D   E   N
CGT GCG AAA GTT GAA ATC ACC CCG AAC TCT CCG CGT GCC GAA GCC ACC CTG GGT GGT TTC
R   A   K   V   E   I   T   P   N   S   P   R   A   E   A   T   L   G   G   F
GGT TCT CTG GGT CTG GAT TGC GAA CCG CGT ACC GGT CTG GAT TTC TCT GAT CTG TAC TAC
G   S   L   G   L   D   C   E   P   R   T   G   L   D   F   S   D   L   Y   Y
CTG ACC ATG AAC AAC AAG CAC TGG CTG GTT CAC AAG GAA TGG TTC CAC GAT ATC CCG CTG
L   T   M   N   N   K   H   W   L   V   H   K   E   W   F   H   D   I   P   L
CCG TGG CAC GCT GGT GCA GAT ACC GGT ACC CCG CAC TGG AAC AAC AAA GAA GCA CTG GTT
P   W   H   A   G   A   D   T   G   T   P   H   W   N   N   K   E   A   L   V
GAA TTC AAA GAT GCA CAC GCC AAA CGT CAG ACC GTT GTT GTT CTG GGT TCT CAG GAA GGT
E   F   K   D   A   H   A   K   R   Q   T   V   V   V   L   G   S   Q   E   G
GCA GTT CAC ACC GCC CTG GCT GGT GCT CTG GAA GCT GAA ATG GAT GGT GCA AAA GGT CGT
A   V   H   T   A   L   A   G   A   L   E   A   E   M   D   G   A   K   G   R
CTG TCT TCT GGT CAC CTG AAA TGC CGT CTG AAA ATG GAT AAA CTG CGT CTG AAA GGT GTT
L   S   S   G   H   L   K   C   R   L   K   M   D   K   L   R   L   K   G   V
TCT TAC TCT CTG TGC ACC GCA GCG TTC ACC TTC ACC AAA ATC CCG GCT GAA ACC CTG CAC
S   Y   S   L   C   T   A   A   F   T   F   T   K   I   P   A   E   T   L   H
GGT ACC GTT ACC GTG GAA CTG CAG TAC GCA GGT ACC GAT GGT CCG TGC AAA GTT CCG GCT
G   T   V   T   V   E   L   Q   Y   A   G   T   D   G   P   C   K   V   P   A
CAG ATG GCG GTG GAT ATG CAG ACC CTG ACC CCG GTT GGT CGT CTG ATC ACC GCT AAC CCG
Q   M   A   V   D   M   Q   T   L   T   P   V   G   R   L   I   T   A   N   P
```

FIGURE 15B

```
GTT ATC ACC GAA TCT ACC GAA AAC TCT AAA ATG ATG CTG GAA CTG GAT CCG CCG TTC GGT
V   I   T   E   S   T   E   N   S   K   M   M   L   E   L   D   P   P   F   G
GAT TCT TAC ATC GTT ATC GGT GTG GGT GAA AAA AAA ATC ACC CAC CAC TGG CAC CGC TCT
D   S   Y   I   V   I   G   V   G   E   K   K   I   T   H   H   W   H   R   S
GGC TCT ACC ATC GGT AAA GCA TTC GAA GCC ACC GTT CGT GGT GCC AAA CGT ATG GCA GTT
G   S   T   I   G   K   A   F   E   A   T   V   R   G   A   K   R   M   A   V
CTG GGT GAT ACA GCC TGG GAT TTC GGT TCT GTT GGT GGT GCT CTG AAC TCT CTG GGT AAA
L   G   D   T   A   W   D   F   G   S   V   G   G   A   L   N   S   L   G   K
GGT ATC CAC CAG ATC TTC GGT GCA GCT TTC AAA TCT CTG TTC GGT GGT ATG TCT TGG TTC
G   I   H   Q   I   F   G   A   A   F   K   S   L   F   G   G   M   S   W   F
TCT CAA ATC CTG ATC GGT ACC CTG CTG ATG TGG CTG GGT CTG AAC ACC AAA AAC GGT TCT
S   Q   I   L   I   G   T   L   L   M   W   L   G   L   N   T   K   N   G   S
ATC TCT CTG ATG TGC CTG GCC CTG GGT GGT GTT CTG ATC TTC CTG TCT ACC GCC GTT TCT
I   S   L   M   C   L   A   L   G   G   V   L   I   F   L   S   T   A   V   S
      NaeI                                   NaeI        XmaI
GCT GCC GGC CAC CAT CAC CAT CAC CAT TAG CCGGCTAATCCCGGG (SEQ ID NO: 37)
A   A   G   H   H   H   H   H   H   *                   (SEQ ID NO: 38)
```

FIGURE 15B (continued)

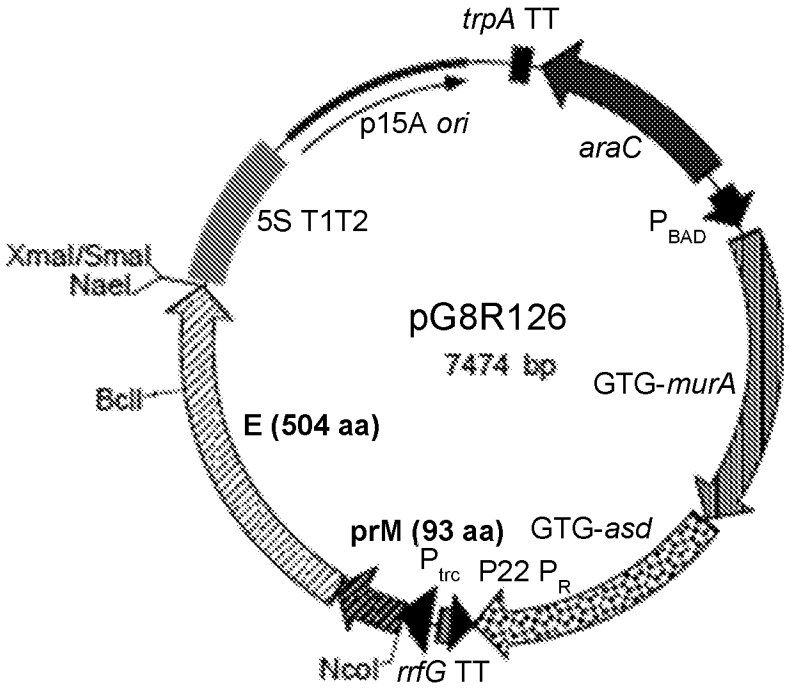

FIGURE 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/43511

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/112, 39/12; C07K 14/005; C12N 1/21 (2017.01)
CPC - A61K 39/0275, 2039/522, 2039/523, C12N 2770/24111, 2770/24134, 2800/22, 15/74; C07K 14/816

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)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Wang et al. New technologies in developing recombinant attenuated Salmonella vaccine vectors. Microb Pathog May 2013 Vol 58 Pg 17-28. Especially pg 18 col 2 para 1-2, pg 19 table 1, pg 21 col 1 para 2, pg 22 col 2 para 4, pg 23 col 1 para 3, pg 24 col 1 para 3	1-4, 7, 8, 26-31
Y	Larocca et al. Vaccine protection against Zika virus from Brazil. Nature ePub 28 June 2016 Vol 536 no 7617 Pages 474-478. Especially abstract, pg 475 fig 1A, online methods.	1-4, 7, 8, 11-13, 26-31
Y	Kong et al. Turning self-destructing Salmonella into a universal DNA vaccine delivery platform. Proc Nat Acad Sci 20 November 2012 Vol 109 No 47 Pages 19414-19419. Especially abstract.	11-13
Y	Barba-Spaeth et al. Structural basis of potent Zika-dengue virus antibody cross-neutralization. Nature ePub 23 June 2016 Vol 536 No 7614 Pages 48-53. Especially pg 48 col 1 para 2; pg 49 col 2 para 1	3, 4, 12, 13, 29-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 2017	12 OCT 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/43511

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

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

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

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

3. ☒ Claims Nos.: 5, 6, 9, 10, 14-25, 32-41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.