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(54) **Titre : PROCÉDES ET COMPOSITIONS POUR TRAITER ET PREVENIR UNE INFECTION A ESCHERICHIA COLI PRODUISANT LA TOXINE SHIGA**
(54) **Title: METHODS AND COMPOSITIONS FOR TREATING AND PREVENTING SHIGA TOXIN-PRODUCING ESCHERICHIA COLI INFECTION**

(57) **Abrégé/Abstract:**

Compositions and methods for stimulating an immune response against Shiga toxin-producing Escherichia coli (STEC) antigens are disclosed. The compositions include a multiple epitope fusion protein comprising more than one epitope of an immunogenic STEC protein from more than one STEC serotype. Additional compositions include at least two purified STEC proteins, wherein the STEC proteins are selected from a full-length STEC protein, an immunogenic fragment or variant thereof, wherein at least one of the STEC proteins generates antibodies that react with STEC 0157 and at least one other STEC serotype.

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(54) **Title:** METHODS AND COMPOSITIONS FOR TREATING AND PREVENTING SHIGA TOXIN-PRODUCING ESCHERICHIA COLI INFECTION

(57) **Abstract:** Compositions and methods for stimulating an immune response against Shiga toxin-producing Escherichia coli (STEC) antigens are disclosed. The compositions include a multiple epitope fusion protein comprising more than one epitope of an immunogenic STEC protein from more than one STEC serotype. Additional compositions include at least two purified STEC proteins, wherein the STEC proteins are selected from a full-length STEC protein, an immunogenic fragment or variant thereof, wherein at least one of the STEC proteins generates antibodies that react with STEC 0157 and at least one other STEC serotype.



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METHODS AND COMPOSITIONS FOR TREATING AND PREVENTING SHIGA
TOXIN-PRODUCING *ESCHERICHIA COLI* INFECTION

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Field of the Invention

10 The present invention relates to compositions and methods for eliciting an immune
response in mammals against Shiga toxin-producing *Escherichia coli* (STEC). In
particular, the invention relates to the use of multiple epitopes from effectors and/or
structural proteins from more than one STEC serotype, as well as epitopes cross-reactive
with more than one serotype, for treating and preventing STEC disease and colonization of
15 mammals.

Background of The Invention

Shiga toxin-producing *Escherichia coli* (STEC), also called Enterohemorrhagic *E.*
coli (EHEC) and verotoxigenic *E. coli* (VTEC) are pathogenic bacteria that cause diarrhea,
20 hemorrhagic colitis, hemolytic uremic syndrome (HUS), kidney failure and death in
humans. Cattle are the primary reservoir for many STEC serotypes and have been
implicated in most disease outbreaks through contamination of food products or the
environment. Many STEC serotypes are capable of causing disease in humans, including,
serotypes O157, O26, O103, O111, among others.

25 STEC organisms colonize the large intestine of cattle and humans by a unique
mechanism in which a number of virulence determinants are delivered to host cells via a
type III secretion system (TTSS), including the translocated Intimin receptor, Tir
(DeVinney et al., *Infect. Immun.* (1999) 67:2389). In particular, these pathogens secrete
virulence determinants EspA, EspB and EspD that enable delivery of Tir into intestinal
30 cell membranes. Tir is integrated into the host cell membrane where it serves as the

receptor for a bacterial outer membrane protein, Intimin. Tir-Intimin binding attaches STEC to the intestinal cell surface and triggers actin cytoskeletal rearrangements beneath adherent STEC that results in pedestal formation. EspA, EspB, Tir and Intimin are each essential for the successful colonization of the intestine by STEC.

5 Although STEC colonize the intestine of ruminants and other mammals, they generally do not cause overt disease in these animals. However, contamination of meat and water by STEC serotypes is responsible for about 50,000 cases of STEC infection in humans annually in the United States and Canada that result in approximately 500 deaths. In 1994, the economic cost associated with STEC infection in humans was estimated to be
10 over 5 billion dollars.

 Healthy ruminants including, but not limited to, cattle, dairy cows and sheep, could be infected with STEC serotypes. In fact, USDA reports indicate that up to 50% of cattle are carriers of STEC at some time during their lifetime and, therefore, shed STEC in their feces.

15 Because of the bulk processing of slaughtered cattle and the low number of STEC (10-100) necessary to infect a human, STEC colonization of healthy cattle remains a serious health problem. To address this problem, research has focused on improved methods for detecting and subsequently killing STEC at slaughter, altering the diet of cattle to reduce the number of intestinal STEC and immunizing animals to prevent STEC
20 colonization (Zacek D. Animal Health and Veterinary Vaccines, Alberta Research Counsel, Edmonton, Canada, 1997). Recently, the recombinant production and use of STEC O157:H7 proteins including recombinant EspA (International Publication No. WO 97/40063), recombinant TIR (International Publication No. WO 99/24576), recombinant EspB and recombinant Intimin (Li et al., *Infec. Immun.* (2000) 68:5090-5095) have been
25 described.

 Babiuk et al., *Microbial Pathogen.* (2008) 45:7-11 describes subcutaneous and intranasal immunization of a mouse model using type III secreted proteins (TTSPs) from STEC serotype O157:H7. U.S. Patent No. 7,300,659 describes the use of cell culture supernatants containing STEC antigens for reducing colonization of STEC. Potter et al.,
30 *Vaccine* (2004) 22:362-369 reports decreased shedding of STEC serotype O157:H7 by

cattle following vaccination with TTSPs. Asper et al., *Vaccine* (2007) 25:8262-8269 examined the cross-reactivity of TTSPs of serotypes O26:H11, O103:H2, O111:NM and O157:H7 and vaccinated cattle with TTSPs produced from each of these serotypes. The authors found the animals responded well with antibodies to TTSPs of the homologous
5 serotype but observed limited cross-reactivity against the other serotypes. No cross-reactivity was observed against Tir and EspA of serotype O157:H7.

Despite the above, there remains a need for new compositions and methods for treating and preventing STEC disease, as well as for reducing STEC colonization of mammals in order to reduce the incidence of health problems associated with
10 STEC-contaminated meat and water.

Summary of The Invention

The present invention satisfies the above need by providing such compositions and methods. In particular, the methods of the present invention make use of compositions
15 including a combination of epitopes from one or more STEC serotypes, as well as epitopes that generate antibodies that cross-reactive with more than one STEC serotype, in order to elicit an immune response against one or more STEC antigens from one or more STEC serotypes, thereby treating and/or preventing STEC infection and/or reducing STEC colonization of the mammal. By providing multiple epitopes derived from more than one
20 serotype, or STEC antigens from at least one serotype that generate cross-reactive antibodies with other STEC serotypes, broad-based protection against diseases caused by STEC can be achieved. The compositions can be delivered with or without a coadministered adjuvant.

Accordingly, it is an object of the present invention to provide a vaccine effective
25 to stimulate an immune response against STEC antigens, thereby treating and/or preventing STEC disease in a mammal.

Another object is to provide a vaccine effective to reduce, prevent and/or eliminate STEC colonization of a ruminant or other mammal.

Another object is to reduce the number of animals shedding STEC into the
30 environment.

Another object is to reduce the number of STEC shed into the environment by an infected animal.

Another object is reduce the time during which STEC are shed into the environment by an infected animal.

5 Another object is reduce STEC contamination of the environment.

Another object is reduce STEC contamination of meat and/or water.

Another object is to treat, prevent and/or reduce STEC infections in humans.

Another object is to provide a vaccine effective as an adjunct to other biological anti-STECC agents.

10 Another object is to provide a vaccine effective as an adjunct to chemical anti-STECC agents.

Another object is to provide a vaccine effective as an adjunct to biologically engineered anti-STECC agents.

15 Another object is to provide a vaccine effective as an adjunct to nucleic acid-based anti-STECC agents.

Another object is to provide a vaccine effective as an adjunct to recombinant protein anti-STECC agents.

Another object is to provide a vaccination schedule effective to reduce STECC colonization of a ruminant.

20 Another object is to provide a vaccination schedule effective to reduce STECC shedding by a ruminant.

Another object is to provide a vaccine effective to prevent, reduce or eliminate STECC O157 colonization of cattle, such as colonization of O157:H7 and/or O157:NM, as well as other members of STECC seropathotypes A and B, such as but not limited to STECC O26, such as O26:H11, STECC O103, such as O103:H2, STECC O111, such as O111:NM, STECC 121:H19, STECC O145:NM, STECC O91:H21, STECC O104:H21 and/or STECC O113:H21.

Another object is to reduce the number of cattle shedding STECC into the environment, such as shedding of O157:H7 and/or O157:NM, as well as other members of STECC seropathotypes A and B, such as but not limited to STECC O26, such as O26:H11,

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STEC O103, such as O103:H2, STEC O111, such as O111:NM, STEC 121:H19, STEC O145:NM, STEC O91:H21, STEC O104:H21 and/or STEC O113:H21.

Another object is to reduce the number of STEC shed into the environment by infected cattle, such as shedding of O157:H7 and/or O157:NM, as well as other members
5 of STEC seropathotypes A and B, such as but not limited to STEC O26, such as O26:H11, STEC O103, such as O103:H2, STEC O111, such as O111:NM, STEC 121:H19, STEC O145:NM, STEC O91:H21, STEC O104:H21 and/or STEC O113:H21.

Another object is reduce the time during which STEC are shed into the environment by infected cattle, such as shedding of O157:H7 and/or O157:NM, as well as
10 other members of STEC seropathotypes A and B, such as but not limited to STEC O26, such as O26:H11, STEC O103, such as O103:H2, STEC O111, such as O111:NM, STEC 121:H19, STEC O145:NM, STEC O91:H21, STEC O104:H21 and/or STEC O113:H21.

Another object is to provide a vaccine effective as an adjunct to other anti-STEC O157, O26, O103, and/or O111 agents, as well as other members of STEC seropathotypes
15 A and B, such as but not limited to STEC 121 STEC O145, STEC O91, STEC O104 and/or STEC O113.

Another object is to provide a vaccination schedule effective to reduce STEC O157, O26, O103, and/or O111 colonization of cattle, as well as colonization of cattle with other
20 members of STEC seropathotypes A and B, such as but not limited to STEC 121 STEC O145, STEC O91, STEC O104 and/or STEC O113.

Another object is to provide a vaccination schedule effective to reduce STEC O157, O26, O103, and/or O111 shedding by cattle, as well as shedding by cattle of other
members of STEC seropathotypes A and B, such as but not limited to STEC 121 STEC O145, STEC O91, STEC O104 and/or STEC O113.

25 Thus, in one embodiment, the invention is directed to a multiple epitope fusion protein comprising more than one epitope of an immunogenic Shiga toxin-producing *Escherichia coli* (STEC) protein from more than one STEC serotype. In certain embodiments, the STEC serotypes are selected from STEC O157, STEC O26, STEC O103 or STEC O111, such as STEC O157:H7, STEC O26:H11, STEC O103:H2 or STEC
30 O111:NM.

In additional embodiments at least one epitope in the multiple epitope fusion protein is derived from STEC O157:H7 Tir. In additional embodiments, the epitopes comprise epitopes derived from STEC O157:H7 Tir, STEC O26:H11 Tir, STEC O103:H2 Tir and STEC O111:NM Tir.

5 In yet further embodiments, the multiple epitope fusion protein comprises a sequence of amino acids at least 80% identical to the sequence of amino acids depicted in Figure 5B, such as a sequence at least 90% identical to the sequence of amino acids depicted in Figure 5B, or even 100% identical to the sequence of amino acids depicted in Figure 5B.

10 In any of the embodiments described above, the multiple epitope fusion protein can be linked to a carrier molecule, such as an RTX toxin. In certain embodiments, the RTX toxin is a leukotoxin polypeptide, such as LKT 352.

In certain embodiments, the protein comprises a sequence of amino acids at least 80% identical to the sequence of amino acids depicted in Figure 6B, such as a sequence at
15 least 90% identical to the sequence of amino acids depicted in Figure 6B, or even 100% identical to the sequence of amino acids depicted in Figure 6B.

In additional embodiments the invention is directed to a composition comprising a multiple epitope fusion protein of any one of the embodiments described above and a pharmaceutically acceptable vehicle.

20 In further embodiments, the invention is directed to a method of producing a composition comprising combining any one of the multiple epitope fusion proteins above with a pharmaceutically acceptable vehicle.

In additional embodiments, the invention is directed to a polynucleotide comprising a coding sequence encoding any one of the multiple epitope fusion proteins
25 above, as well as a recombinant vector comprising the polynucleotide and control elements that are operably linked to the polynucleotide whereby said coding sequence can be transcribed and translated in a host cell. In further embodiments, the invention is directed to a host cell transformed with the recombinant vector, as well as methods of producing a multiple epitope fusion protein comprising providing a population of the host cells and
30 culturing said population of cells under conditions whereby the protein encoded by the

coding sequence present in the recombinant vector is expressed.

In further embodiments, the invention is directed to antibodies specific for any one of the multiple epitope fusion proteins above, such as but not limited to polyclonal or monoclonal antibodies.

5 In additional embodiments, the invention is directed to methods of detecting STEC antibodies in a biological sample comprising providing a biological sample; reacting the biological sample with any one of the multiple epitope fusion proteins above under conditions which allow STEC antibodies, when present in the biological sample, to bind to the multiple epitope fusion protein to form an antibody/antigen complex; and detecting the
10 presence or absence of the complex, thereby detecting the presence or absence of STEC antibodies in the sample.

In further embodiments, the invention is directed to an immunodiagnostic test kit for detecting STEC infection, the test kit comprising any one of the multiple epitope fusion proteins above, and instructions for conducting the immunodiagnostic test.

15 In other embodiments, the invention is directed to a composition comprising at least two purified immunogenic Shiga toxin-producing *Escherichia coli* (STEC) proteins, wherein the STEC proteins are selected from a full-length STEC protein, an immunogenic fragment or variant thereof, wherein at least one of the STEC proteins generates antibodies that react with STEC O157 and at least one other STEC serotype. In certain embodiments,
20 at least one of the STEC proteins generates antibodies that react with STEC O157 and at least two and/or three or more other STEC serotypes.

In additional embodiments, the composition comprises more than one STEC protein selected from Tir, EspA, EspB, EspD, NleA, Tccp, EspG, NleE and NleH. In certain embodiments, the STEC proteins are from STEC O157:H7.

25 In further embodiments, the compositions above further comprise any one of the multiple epitope fusion proteins described above.

In certain embodiments, the compositions described above comprise an immunological adjuvant.

In additional embodiments, the invention is directed to a method for eliciting an
30 immunological response in a mammal against a STEC antigen, the method comprising

administering to the mammal a therapeutically effective amount of any one of the compositions described above. In certain embodiments, the mammal is a ruminant, such as a bovine subject.

In yet further embodiments, the invention is directed to a method for reducing
5 colonization of STEC in a ruminant, and/or a method for reducing shedding of STEC from a ruminant, comprising administering to the ruminant a therapeutically effective amount of any one of the compositions described above.

Brief Description of the Drawings

10 Figures 1A-1B (SEQ ID NOS:44 and 45) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 Tir.

Figures 2A-2B (SEQ ID NOS:46 and 47) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O26:H11 Tir.

15 Figures 3A-3B (SEQ ID NOS:217 and 48) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O103:H2 Tir.

Figures 4A-4B (SEQ ID NOS:49 and 50) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O111:NM Tir.

Figures 5A-5B (SEQ ID NOS:51 and 52) show the nucleotide sequence and amino acid sequence, respectively, for a representative chimeric Tir construct.

20 Figures 6A-6B (SEQ ID NOS:53 and 54) show the nucleotide sequence and amino acid sequence, respectively, for a representative chimeric Tir construct fused to a leukotoxin carrier.

Figure 7 shows the reactivity of STEC O157:H7 peptides with rabbit antisera raised against STEC O157:H7, O26:H11, O103:H2 and O111:NM TTSPs.

25 Figures 8A-8D show the cross-reactivity of STEC polyclonal antibodies raised against STEC O157:H7, O26:H11, O103:H2 and O111:NM TTSPs with O103:H2 Tir peptides (8A); O26:H11 Tir peptides (8B); O111:NM Tir peptides (8C); and O157:H7 Tir peptides (8D).

30 Figures 9A-9C show the cloning scheme used for the construction of a representative chimeric Tir protein. Figure 9A shows the individual fragments cloned

including restriction sites and the location of the spacers composed of Gly and Ser residues. Figure 9B shows a diagram of a representative chimeric Tir construct. Figure 9C shows a diagram of a representative chimeric Tir construct fused to a leukotoxin LKT 352 carrier.

Figure 10 depicts the structure of Plasmid pAA352 wherein tac is the hybrid
 5 trp::lac promoter from *E. coli*; bla represents the β -lactamase gene (ampicillin resistance); ori is the ColEI-based plasmid origin of replication; lktA is the *P. haemolytica* leukotoxin structural gene; and lacI is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

10 Figures 11A-11I (SEQ ID NOS:55, 56 and 218) show the nucleotide sequence and predicted amino acid sequence of leukotoxin 352 (LKT 352) from plasmid pAA352. Both the structural gene for LKT 352 and the sequences of the flanking vector regions are shown.

Figures 12A-12J show ELISA results using sera from rabbits vaccinated with
 15 chimeric Tir proteins and individual non-O157 immunogenic peptides. Figure 12A shows the titer results against the chimeric Tir protein. Figure 12B shows the titer results against the LKT 352/chimeric Tir protein. Figures 12C-12H show the titer results against individual non-O157 peptides from Table 2 of the Examples as follows; Figure 12C, O26 Peptide 2; Figure 12D, O26 Peptide 3; Figure 12E, O103 Peptide 5; Figure 12F, O111
 20 Peptide 3; Figure 12G, O111 Peptide 4; Figure 12H, O111, Peptide 5. Figure 12I shows the titer results against the negative control Peptide SN11. Figure 12J shows the titer results against the Tir protein from STEC O157:H7.

Figure 13 shows the antibody response of sera from STEC O157:H7
 experimentally infected cattle against STEC O157 secreted proteins. Animal 1 is
 25 represented by the grey bars. Animal 2 is represented by the stippled bars.

Figure 14 shows the results of ELISAs using Walkerton natural infected human serum samples against STEC O157:H7 Tir antigen.

Figure 15 shows the antibody response of human sera from HUS patients against STEC O157 secreted proteins.

30 Figures 16A and 16B (SEQ ID NOS:197 and 198) show the nucleotide sequence

and amino acid sequence, respectively, for a representative STEC O157:H7 EspA.

Figures 17A and 17B (SEQ ID NOS:199 and 200) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 EspB.

Figures 18A and 18B (SEQ ID NOS:201 and 202) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 EspD.

Figures 19A and 19B (SEQ ID NOS:203 and 204) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 NleA.

Figures 20A and 20B (SEQ ID NOS:205 and 206) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 EspG.

Figures 21A and 21B (SEQ ID NOS:207 and 208) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 NleE.

Figures 22A and 22B (SEQ ID NOS:209 and 210) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 NleH-1.

Figures 23A and 23B (SEQ ID NOS:211 and 212) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 NleH2-1.

Figures 24A and 24B (SEQ ID NOS:213 and 214) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 EspF.

Figures 25A and 25B (SEQ ID NOS:215 and 216) show the nucleotide sequence and amino acid sequence, respectively, for a representative STEC O157:H7 EspRI.

Figure 26 shows amount of *E. coli* O157 fecal shedding in mice treated with placebo (■); O157 TTSPs (▲) and a mixture of recombinant O157:H7 EspG, NleH2-1, NleA, EspRI, EspF, EspB, EspD, EspA and the chimeric Tir (▼).

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Vols. I, II and III, Second Edition (1989); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S.

Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

5

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

10 It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a STEC bacterium " includes a mixture of two or more such bacteria, and the like.

As used herein, the term STEC "effector protein" or a nucleotide sequence
15 encoding the same, intends a protein or a nucleotide sequence, respectively, which is derived from any of the various STEC serotypes and which is translocated by the locus for enterocyte effacement (LEE) pathogenicity island. This locus encodes the Esc-Esp type III secretion system which is crucial to the virulence of STEC bacteria. Effector proteins, however, can be encoded either within or outside of the LEE pathogenicity island.
20 Multiple STEC effector proteins are known and various sequences are described herein and in the art. See, e.g., Tobe et al., *Proc. Natl. Acad. Sci. USA* (2006) 103:14941-14946, as well as the disclosure herein, for a discussion of both LEE and non-LEE STEC effector proteins. Non-limiting examples of STEC effector proteins include Tir, NleA, TccP, EspM2 and EspB.

25 As used herein, the term STEC "structural protein" or a nucleotide sequence encoding the same, intends a protein or a nucleotide sequence, respectively, which is derived from any of the various STEC serotypes and which is part of the physical complex necessary for the secretion of effector proteins into the cell. Structural proteins are usually found in association with the bacterial cell. Examples of such structural proteins
30 include needle components, such as the base and tip of the needle; outer membrane

components and filament components. A number of STEC structural proteins are known and the sequences are described herein and in the art. Non-limiting examples of STEC structural proteins include EspA and EspD.

As used herein, a "recombinant" STEC protein, such as, but not limited to, rTir,
5 rEspA, rEspB, rEspD, rEspF, rEspG, rEspRI, rNleA, rNleH2-1, rEspM2 and rTccp, as well as rIntimin, means a protein produced by expression of a recombinant polynucleotide. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions. A "recombinant" protein refers to the full-length
10 polypeptide sequence, fragments of the reference sequence or substitutions, deletions and/or additions to the reference sequence, so long as the proteins retain at least one specific epitope or activity. Generally, analogs of the reference sequence will display at least about 50% sequence identity, preferably at least about 75% to 85% sequence identity, and even more preferably about 90% to 95% or more sequence identity, to the full-length
15 reference sequence.

By the term "multiple epitope fusion protein" is meant a protein including more than one epitope of a STEC effector and/or structural protein, wherein the epitopes are not found in the order they are found in nature. Thus, a multiple epitope fusion protein includes more than one repeat of the same epitope, as well as more than one epitope from
20 the same protein, or more than one epitope from more than one protein. The epitopes need not be directly connected to each other, are not repeated in nature in the same manner and, further, may be present within a larger sequence which includes other amino acids that are not STEC epitopes. For the purposes of this invention, the epitope sequences present in the fusion may either be an exact copy of a wild-type epitope sequence, or a
25 sequence which is "functionally equivalent" thereto, i.e., one that will elicit a substantially equivalent or enhanced immunological response, as defined herein, as compared to the response elicited by an epitope having identity with either the full-length molecule from which the epitope is derived, or an immunogenic portion thereof. Additionally, multiple epitope fusion proteins may include the full-length molecules, or immunogenic fragments
30 thereof.

The terms “polypeptide” and “protein” refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include
5 postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a “polypeptide” refers to the native protein sequence, as well as a protein which includes modifications, such as deletions, additions and substitutions, to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate,
10 as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

The term “peptide” as used herein refers to a fragment of a polypeptide. Thus, a peptide can include a C-terminal deletion, an N-terminal deletion and/or an internal deletion of the native polypeptide, so long as the entire protein sequence is not present. A
15 peptide will generally include at least about 3-10 contiguous amino acid residues of the full-length molecule, and can include at least about 15-25 contiguous amino acid residues of the full-length molecule, or at least about 20-50 or more contiguous amino acid residues of the full-length molecule, or any integer between 3 amino acids and the number of amino acids in the full-length sequence, provided that the peptide in question retains the ability to
20 elicit the desired biological response.

A STEC “peptide” is a polypeptide that includes less than the full-length sequence of a STEC protein. Moreover, a STEC peptide will include at least one epitope such that an immunologic response can be generated. A STEC peptide can be derived from any of the various STEC serotypes, as described below.

25 As used herein, “vaccine” refers to a composition that serves to stimulate an immune response to a STEC antigen, such as a STEC effector and/or structural protein. The immune response need not provide complete protection and/or treatment against STEC infection or against colonization and shedding of STEC. Even partial protection against colonization and shedding of STEC bacteria will find use herein as shedding and
30 contaminated meat production will still be reduced. In some cases, a vaccine will include

an immunological adjuvant in order to enhance the immune response. The term “adjuvant” refers to an agent which acts in a nonspecific manner to increase an immune response to a particular antigen or combination of antigens, thus reducing the quantity of antigen necessary in any given vaccine, and/or the frequency of injection necessary in order to generate an adequate immune response to the antigen of interest. See, e.g., A.C. Allison *J. Reticuloendothel. Soc.* (1979) 26:619-630. Such adjuvants are described further below.

As used herein, “colonization” refers to the presence of STEC in the intestinal tract of a mammal, such as a ruminant.

As used herein, “shedding” refers to the presence of STEC in feces.

As used herein, “immunization” or “immunize” refers to administration of a STEC composition, in an amount effective to stimulate the immune system of the animal to which the composition is administered, to elicit an immunological response against one or more of the antigens present in the composition.

The term “epitope” refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with “antigenic determinant” or “antigenic determinant site.” Preferably an epitope is a short peptide derived from or as part of a protein antigen. Several different epitopes may be carried by a single antigenic molecule. The term “epitope” also includes modified sequences of amino acids which stimulate responses which recognize the whole organism. The epitope can be generated from knowledge of the amino acid and corresponding DNA sequences of the peptide or polypeptide, as well as from the nature of particular amino acids (e.g., size, charge, etc.) and the codon dictionary, without undue experimentation. See, e.g., Ivan Roitt, *Essential Immunology*, 1988; Kendrew, *supra*; Janis Kuby, *Immunology*, 1992 e.g., pp. 79-81.

An “immunological response” to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, an “immunological response” includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an

antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that STEC disease is lessened and/or prevented; resistance of the intestine to colonization with STEC is imparted; the number of animals shedding STEC is reduced; the number of STEC shed by an animal is reduced; and/or the time period of STEC shedding by an animal is reduced.

The terms "immunogenic" protein or polypeptide refer to an amino acid sequence which elicits an immunological response as described above. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the particular STEC protein in question, analogs thereof, aggregates, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a STEC protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in *Methods in Molecular Biology*, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715.

Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra*. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omega version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

Immunogenic fragments, for purposes of the present invention, will usually include

at least about 3 amino acids, preferably at least about 5 amino acids, more preferably at least about 10-15 amino acids, and most preferably 25 or more amino acids, of the parent STEC protein molecule. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion
 5 protein comprising two or more epitopes of the particular STEC protein.

An "antigen" refers to a molecule, such as a protein, polypeptide, or fragment thereof, containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune-system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Antibodies such as anti-idiotypic
 10 antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide which expresses an antigen or antigenic determinant *in vivo*, such as in DNA immunization applications, is also included in the definition of antigen herein.

15 By "carrier" is meant any molecule which when associated with an antigen of interest, imparts immunogenicity to the antigen.

The term "RTX" toxin, as used herein refers to a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (SEQ ID NO:219) (Highlander et al., *DNA* (1989) 8:15-28), where
 20 X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35; Welch, *Mol. Microbiol.* (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35). In
 25 addition, the term "RTX toxin" refers to a member of the RTX family which is chemically synthesized, isolated from an organism expressing the same, or recombinantly produced. Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native RTX molecule. Thus, the term includes both full-length and partial sequences, as
 30 well as analogues. Although native full-length RTX toxins display cytotoxic activity, the term "RTX toxin" also intends

molecules which remain immunogenic yet lack the cytotoxic character of native molecules. In the chimeras produced according to the present invention, a selected RTX polypeptide sequence imparts enhanced immunogenicity to a fused STEC protein or multiple epitope fusion protein.

5 The term “leukotoxin polypeptide” or “LKT polypeptide” intends an RTX toxin derived from *P. haemolytica*, *Actinobacillus pleuropneumoniae*, among others, as defined above. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al., *Infect. Immun.* (1985) 50:667-67; Lo et al., *Infect. Immun.* (1987) 55:1987-1996; Strathdee
10 et al., *Infect. Immun.* (1987) 55:3233-3236; Highlander et al., *DNA* (1989) 8:15-28; Welch, *Mol. Microbiol.* (1991) 5:521-528. A selected leukotoxin polypeptide sequence imparts enhanced immunogenicity to a fused STEC protein or multiple epitope fusion protein.

 A STEC protein that is linked to a carrier displays "enhanced immunogenicity" when it possesses a greater capacity to elicit an immune response than the corresponding
15 protein alone. Such enhanced immunogenicity can be determined by administering the particular protein/carrier complex and protein controls to animals and comparing antibody titers against the two using standard assays such as radioimmunoassays and ELISAs, well known in the art.

 The term “purified” refers to isolation of a substance (compound, polynucleotide,
20 protein, polypeptide, polypeptide composition) such that the substance comprises the majority percent of the sample in which it resides. Typically in a sample, a purified component comprises 50%, preferably 80%-85%, more preferably 90-95% of the sample. Expressly excluded from the definition of purified herein is a component of a cell culture supernatant which contains a mixture of STEC antigens that have been secreted into the
25 growth media, such as described in U.S. Patent No. 7,300,659. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

 By “isolated” is meant, when referring to a polypeptide, that the indicated molecule
30 is separate and discrete from the whole organism with which the molecule is found in

nature or is present in the substantial absence of other biological macro-molecules of the same type. The term “isolated” with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

An “antibody” intends a molecule that “recognizes,” i.e., specifically binds to an epitope of interest present in an antigen. By “specifically binds” is meant that the antibody interacts with the epitope in a “lock and key” type of interaction to form a complex between the antigen and antibody, as opposed to non-specific binding that might occur between the antibody and, for instance, components in a mixture that includes the test substance with which the antibody is reacted. Thus, for example, an anti-STEC effector antibody is a molecule that specifically binds to an epitope of the STEC effector protein in question. The term “antibody” as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter et al., *Nature* (1991) 349:293-299; and U.S. Patent No. 4,816,567); F(ab')₂ and F(ab) fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar et al., *Proc Natl Acad Sci USA* (1972) 69:2659-2662; and Ehrlich et al., *Biochem* (1980) 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al., *Proc Natl Acad Sci USA* (1988) 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al., *Biochem* (1992) 31:1579-1584; Cumber et al., *J Immunology* (1992) 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al., *Nature* (1988) 332:323-327; Verhoeyan et al., *Science* (1988) 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

As used herein, the term “monoclonal antibody” refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made.

The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')₂,

Fv, and other fragments, as well as chimeric and humanized homogeneous antibody populations, that exhibit immunological binding properties of the parent monoclonal antibody molecule.

“Native” proteins or polypeptides refer to proteins or polypeptides isolated from the source in which the proteins naturally occur. “Recombinant” polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide.

“Synthetic” polypeptides are those prepared by chemical synthesis.

“Homology” refers to the percent identity between two polynucleotide or two polypeptide moieties. Two nucleic acid, or two polypeptide sequences are “substantially homologous” to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified sequence.

In general, “identity” refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules (the reference sequence and a sequence with unknown % identity to the reference sequence) by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the reference sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters

recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide
5 positions.

Another method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the
10 Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with
15 default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs are readily
20 available.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a
25 Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

"Recombinant" as used herein to describe a nucleic acid molecule means a
30 polynucleotide of genomic, cDNA, viral, semisynthetic, or synthetic origin which, by

virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature.

The term "transformation" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion. For example, direct uptake, transduction or f-mating are included. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Recombinant host cells", "host cells," "cells", "cell lines," "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transferred DNA, and include the original progeny of the original cell which has been transfected.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences (or "control elements"). The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral or procaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

Typical "control elements," include, but are not limited to, transcription promoters, transcription enhancer elements, transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), and translation termination sequences.

A "nucleic acid" molecule can include, but is not limited to, prokaryotic sequences, eucaryotic mRNA, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper enzymes are present. The promoter need not be contiguous
5 with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Encoded by" refers to a nucleic acid sequence which codes for a polypeptide
10 sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences which are immunologically identifiable with a polypeptide encoded by the sequence.

15 The term "transfection" is used to refer to the uptake of foreign DNA by a cell. A cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, e.g., Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (1989) *Molecular Cloning, a laboratory manual*, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) *Basic*
20 *Methods in Molecular Biology*, Elsevier, and Chu et al. (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells. The term refers to both stable and transient uptake of the genetic material, and includes uptake of peptide- or antibody-linked DNAs.

A "vector" is capable of transferring gene sequences to target cells (e.g., viral
25 vectors, non-viral vectors, particulate carriers, and liposomes). Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include fluorescein, rhodamine, dansyl, umbelliferone, Texas red, luminol, NADPH and α - β -galactosidase.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy). Treatment also encompasses the prevention or reduction of STEC colonization of a mammal such as a ruminant; and/or the reduction in the number of STEC shed by an animal; and/or, reducing the time period of STEC shedding by an animal.

As used herein, "therapeutic amount", "effective amount" and "amount effective to" refer to an amount of vaccine effective to elicit an immune response against a STEC antigen present in a composition, thereby reducing or preventing STEC disease, and/or STEC colonization of a mammal such as a ruminant; and/or reducing the number of animals shedding STEC; and/or reducing the number of STEC shed by an animal; and/or, reducing the time period of STEC shedding by an animal.

By "mammalian subject" is meant any member of the class Mammalia, including humans and all other mammary gland possessing animals (both male and female), such as ruminants, including, but not limited to, bovine, porcine and *Ovis* (sheep and goats) species. The term does not denote a particular age. Thus, adults, newborns, and fetuses

are intended to be covered.

B. General Methods

Central to the present invention is the discovery that multiple epitope fusion
5 proteins including more than one STEC epitope from more than one STEC serotype,
produce an immune response in animals to which they are administered. Moreover,
epitopes from STEC effector and structural proteins that generate antibodies that react with
proteins from more than one STEC serotype have been discovered. The chimeric
constructs and cross-reactive STEC proteins are used in vaccine compositions to provide
10 broad-based protection and treatment of STEC infection, such as protection against
colonization. Thus, epitopes derived from various STEC effector and structural proteins
from multiple STEC serotypes will find use in the present compositions and methods.
Such epitopes can be provided individually in one or more subunit vaccine compositions,
or can be conveniently provided as a chimeric protein, expressed recombinantly as a fusion
15 protein or expressed individually and subsequently fused.

In certain embodiments, the compositions comprise a multiple epitope fusion
protein including more than one epitope from more than one STEC serotype, such as
multiple epitopes of Tir from multiple STEC serotypes. In other embodiments, the
compositions comprise a mixture of purified STEC effector and/or structural proteins,
20 which proteins generate antibodies that react with proteins from more than one STEC
serotype, such as, but not limited to STEC proteins selected from EspA, EspB, EspD,
EspG, EspF, EspRI, NleA, NLeH2-1, Tccp, Tir and/or a multiple epitope fusion protein
such as a protein with multiple Tir epitopes.

In some embodiments, the STEC constructs or purified STEC proteins are linked
25 to carrier molecules to enhance immunogenicity. A pharmaceutically acceptable adjuvant
may also be administered with the compositions. The compositions are administered in
an amount effective to elicit an immune response to one or more of the antigens, thereby
reducing or eliminating STEC infection. In some instances, STEC colonization of the
animal is reduced or eliminated. In preferred embodiments, the animal is a cow or a
30 sheep or other ruminant.

Immunization with the compositions of the invention stimulates the immune system of the immunized animal to produce antibodies against one or more STEC antigens, such as EspA, EspB, EspD, EspG, EspF, EspRI, NleA, NLeH2-1, Tccp and/or Tir, that block STEC attachment to intestinal epithelial cells, interfere with STEC colonization and, thereby, reduce STEC shedding by the animal. This reduction in STEC shedding results in a reduction in STEC contamination of food and water and a reduction in STEC-caused disease in humans. Moreover, the ability of immunization to prevent, reduce and eliminate STEC colonization and shedding by cattle addresses a long-felt unfulfilled need in the medical arts, and provides an important benefit for humans.

Additionally, the compositions of the present invention can be used to treat or prevent STEC infections in other mammals such as humans. The use of purified antigens, such as recombinantly produced proteins, allows control of the antigens present, e.g., compositions that lack one or both of the Shiga toxins 1 and 2 in order to reduce toxicity.

The therapeutic effectiveness of the STEC compositions can be enhanced by using natural or synthetic carriers, adjuvants and/or by administering the compositions before, at the same time as, or after another anti-STEC agent. Such agents include, but are not limited to, biological, biologically engineered, chemical, nucleic acid based and recombinant protein anti-STEC agents.

In order to further an understanding of the invention, a more detailed discussion is provided below regarding the STEC proteins and chimeras, production thereof, compositions comprising the same, and methods of using such compositions in the treatment or prevention of infection, as well as in the diagnosis of infection.

I. Polypeptides for use in Chimeric Constructs and Combination Vaccines

As explained above, the proteins of the present invention provide broad protection against more than one STEC serotype by virtue of the use of chimeric constructs including more than one epitope from one or more STEC effector and/or structural proteins from more than one serotype. In alternative embodiments, compositions can include purified STEC proteins, immunogenic fragments and/or variants thereof, that generate antibodies that react with antigens from more than one STEC serotype.

Proteins and epitopes for use with the present invention may be obtained from any of the various STEC serotypes, including, without limitation, STEC serotypes from serogroups O157, O158, O5, O8, O18, O26, O45, O48, O52, O55, O75, O76, O78, O84, O91, O103, O104, O111, O113, O114, O116, O118, O119, O121, O125, O28, O145, O146, O163, O165. Such STEC serotypes are readily obtained from sera of infected animals. Methods for isolating STEC are well known in the art. See, e.g., Elder et al., *Proc. Natl. Acad. Sci. USA* (2000) 97:2999; Van Donkersgoed et al., *Can. Vet. J.* (1999) 40:332; Van Donkersgoed et al., *Can. Vet. J.* (2001) 42:714. Generally, such methods entail direct plating on sorbitol MacConkey agar supplemented with cefixime and tellurite or immunomagnetic enrichment followed by plating on the same media. Moreover, STEC proteins and epitopes may be obtained from STEC serotypes that have been genetically engineered to knock-out expression of Shiga toxins 1 and/or 2, in order to reduce toxicity.

Proteins from which multiple epitope fusion proteins and compositions comprising STEC proteins can include any of various STEC structural proteins, as well as any of the known LEE and non-LEE effectors. Such proteins, include without limitation, EspA, EspD, Tir, NleA, EspB, TccP, Ler, Orf2, CesA/B, Orf4, Orf5, EscS, EscT, Rorfl3, GrlR, GrlA, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16, SepQ, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, EspG, NleB, NleB2-1, NleC, NleE, NleF, NleG, NleH, NleH1-2, NleH2-1, NleI, NleG2-1, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspM2, EspR1, EspV, EspW, EspX2, EspX7, EspY1, EspY2 and EspY3.

The sequences for various STEC proteins are known and/or described herein. See, e.g., GenBank Accession Nos. AE005594, AE005595, AP002566, AE005174, NC_002695, NC_002655, as well as U.S. Patent No. 6,855,814,

for the complete sequence of the *E. coli* O157:H7 genome, which includes the sequences of the various O157:H7 structural and effector proteins; see GenBank Accession Nos. AJ277443 and AJ303141, for the sequences of the LEE pathogenicity islands of STEC O26:H11 and O103:H2, respectively, which include the sequences of various STEC proteins; see GenBank Accession No. AF025311 for the

sequences of STEC O111:H tir, intimin and a chaperone.

See, e.g., International Publication No. WO 97/40063, as well as GenBank
Accession Nos. AE005174, Y13068, U80908, U5681, Z54352, AJ225021, AJ225020,
AJ225019, AJ225018, AJ225017, AJ225016, AJ225015, AF022236, AF200363,
5 NC_011601, NC_002695, BA000007 and AJ303141 for the nucleotide and amino acid
sequences of EspA from a number of *E. coli* serotypes. Figures 16A-16B show the
nucleotide sequence and amino acid sequence, respectively, of a representative STEC
O157:H7 EspA.

See, e.g., Figures 1A-1B for the nucleotide sequence and amino acid sequence,
10 respectively, for STEC O157:H7 Tir; Figures 2A-2B for the nucleotide sequence and
amino acid sequence, respectively, for STEC O26:H11 Tir; Figures 3A-3B for the
nucleotide sequence and amino acid sequence, respectively, for STEC O103:H2 Tir;
Figures 4A-4B for the nucleotide sequence and amino acid sequence, respectively, for
STEC O111:NM Tir; as well as International Publication No. WO 99/24576, as well as
15 GenBank Accession Nos. AF125993, AF132728, AF045568, AF022236, AF70067,
AF070068, AF013122, AF200363, AF113597, AF070069, AB036053, AB026719, U5904
and U59502, for the nucleotide and amino acid sequences of Tir from a number of *E. coli*
serotypes.

See, e.g., GenBank Accession Nos. U32312, U38618, U59503, U66102,
20 AF081183, AF081182, AF130315, AF339751, AJ308551, AF301015, AF329681,
AF319597, AJ275089-AJ275113 for the nucleotide and amino acid sequences of Intimin
from a number of *E. coli* serotypes.

See, e.g., GenBank Accession Nos. AE005174, U80796, U65681, Y13068,
Y13859, X96953, X99670, X96953, Z21555, AF254454, AF254455, AF254456,
25 AF254457, AF054421, AF059713, AF144008, AF144009, NC_011601, NC_002695,
BA000007 and AJ303141 for the nucleotide and amino acid sequences of EspB from a
number of *E. coli* serotypes. Figures 17A-17B show the nucleotide sequence and amino
acid sequence, respectively, of a representative STEC O157:H7 EspB.

See, e.g., GenBank Accession Nos. AE005174, Y13068, Y13859, Y17875,
30 Y17874, Y09228, U65681, AF054421, AF064683, NC_011601, NC_002695, BA000007

and AJ303141 for the nucleotide and amino acid sequences of EspD from a number of *E. coli* serotypes. Figures 18A-18B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 EspD.

See, e.g., GenBank Accession Nos. AE005174, BAF9651, CAM11325, CAM11324, CAM11323, CAM11322, CAM11321, CAM11320, CAM11319, CAM11318, CAM11317, CAM11316, CAM11315, CAM11314, CAM11313 and NC_011601 for the sequences of NleA from a number of *E. coli* serotypes. Figures 19A-19B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 NleA.

See, e.g., GenBank Accession Nos. AE005174, AB356000, AB355999, AB355998, AB355997, AB355996, AB355995, AB355659, AB253549, AB253548, AB253547, AB253546, AB253545, AB253544, AB253543, AB253542, AB253541, AB253540, AB253539, AB253538, AB253537, DQ206456, for the sequences of Tccp from a number of *E. coli* serotypes.

See, e.g., GenBank Accession Nos. AE005174, NC_011601, NC_002695, BA000007 and AJ303141 for the sequences of EspG, NleE and NleH from a number of *E. coli* serotypes. Figures 20A-20B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 EspG. Figures 21A-21B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 NleE. Figures 22A-22B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 NleH1-1.

See, e.g., GenBank Accession Nos. AF022236; AJ303141; NP_290250.1; YP_002331392.1; NP_310742.1; AAG58814.1 for the nucleotide and amino acid sequences of EspF from a number of *E. coli* serotypes. Figures 24A-24B show the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 EspF.

See, e.g., Figures 23A-23B for the nucleotide sequence and amino acid sequence, respectively, of a representative STEC O157:H7 NleH2-1. See, e.g., Figures 25A-25B for a representative STEC O157:H7 EspRI.

Cross-reactive epitopes for use in the compositions as well as epitopes for use in

the chimeras of the present invention can be readily identified by aligning the sequences of STEC proteins from, e.g., two or more of the STEC serotypes listed above, and searching for the variable and conserved regions. Normally, it is desirable to include epitopes from the variable regions of the STEC molecules in order to confer broad-based protection
5 against a variety of bacteria. Useful epitopes can also be identified, e.g., in non-O157 STEC serotypes which have diverged from STEC O157, but that are still recognized by the host immune system. For example, in the case of Tir, portions spanning amino acids 259 to 363 are of particular interest as these amino acids have been shown to be exposed on the surface of the host's epithelial cells, making them a prime target for vaccine development.

10 Additional epitopes can be identified using techniques well known in the art, such as using standard antigenicity and hydropathy plots, for example those calculated using, e.g., the Omega version 1.0 software program available from the Oxford Molecular Group.

This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle
15 technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots. This program can be used with the following parameters: averaging results over a window of 7; determining surface probability according to Emini; chain flexibility according to Karplus-Schulz; antigenicity index according to Jameson-Wolf; secondary structure according to Garnier-Osguthorpe-Robson; secondary structure according to Chou-Fasman;
20 and identifying predicted glycosylation sites. One of skill in the art can readily use the information obtained in combination with teachings of the present specification to identify antigenic regions which may be employed in the compositions of the invention.

In particularly preferred embodiments, compositions contain STEC proteins or immunogenic fragments thereof that generate antibodies that react with STEC O157, such
25 as STEC O157:H7 and/or O157:NM, and at least one other STEC serotype, preferably at least two other STEC serotypes and even more preferably at least three other STEC serotypes, such as STEC O26, e.g., O26:H11, STEC O103, such as O103:H2 and/or STEC O111, such as O111:NM, or any of the STEC serotypes described above, in addition to STEC O157. As described in the examples, each of Tir, EspA, EspB, EspD, NleA and
30 Tccp from STEC O157:H7 generate antibodies that react with STEC O157:H7, as well as

STEC O26:H11, STEC O103:H2 and STEC O111:NM (see Table 5). Additionally, each of EspG, NleE and NleH from STEC O157:H7 generate antibodies that react with STEC O157:H7, as well as STEC O103:H2 and STEC O111:NM (see Table 5).

In certain embodiments, the invention is directed to multiple epitope fusion proteins that include more than one epitope from one or more STEC effector and/or structural proteins. The epitopes can be from the same *E. coli* STEC serotype, or preferably, from multiple STEC serotypes. Additionally, the epitopes can be derived from the same STEC protein or from different STEC proteins from the same or different STEC serotypes.

More particularly, the chimeras may comprise multiple epitopes, a number of different STEC proteins from the same or different serotype, as well as multiple or tandem repeats of selected STEC sequences, multiple or tandem repeats of selected STEC epitopes, or any conceivable combination thereof. Epitopes may be identified using techniques as described above, or fragments of STEC proteins may be tested for immunogenicity and active fragments used in compositions in lieu of the entire polypeptide, as described in the examples. The epitopes may be separated by spacers. The strategic use of various spacer sequences between selected STEC polypeptides can confer increased immunogenicity on the subject constructs. Accordingly, under the invention, a selected spacer sequence may encode a wide variety of moieties of one or more amino acids in length. Selected spacer groups may also provide enzyme cleavage sites so that the expressed chimera can be processed by proteolytic enzymes *in vivo* (by APC's or the like) to yield a number of peptides. Additionally, spacer sequences may be constructed so as to provide T-cell antigenicity, such as those sequences which encode amphipathic and/or α -helical peptide sequences which are generally recognized in the art as providing immunogenic helper T-cell epitopes. If included, the choice of particular T-cell epitopes to be provided by such spacer sequences may vary depending on the particular species to be vaccinated.

Particularly preferred are amino acid spacer sequences. Such spacers will typically include from 1-500 amino acids, preferably 1-100 amino acids, more preferably 1-50 amino acids, preferably 1-25 amino acids, and most preferably 1-10 amino acids, or any integer between 1-500. The spacer amino acids may be the same or different between

various epitopes. Particularly preferred amino acids for use as spacers are amino acids with small side groups, such as serine, alanine, glycine and valine.

Although particular chimeras are exemplified herein which include spacer sequences, it is also to be understood that one or more of the epitopes present in the fusion constructs can be directly adjacent to another epitope, without an intervening spacer sequence.

The nucleotide and amino acid sequences of a particular STEC multiple epitope fusion protein is shown in Figures 5A and 5B (SEQ ID NOS:51 and 52), respectively, and a diagrammatic representation of the sequence is shown in Figure 9B. As shown in Figures 9A and 9B, this protein includes epitopes derived from the effector protein Tir from four different STEC serotypes. The DNA sequence includes the full-length coding sequence for STEC O157:H7, as well as 240 basepairs of STEC O111:NM Tir, 165 basepairs of STEC O26:H11 Tir and 90 basepairs of O103:H2 Tir. These sequences are separated by spacers comprised of various combinations of the amino acids Gly and Ser.

The protein includes in N-terminal to C-terminal order the full-length O157 Tir sequence (amino acids 1 to 558 of Figure 5B), followed by the linker Gly-Ser-Gly-Ser (SEQ ID NO: 220), followed by amino acids 279 to 358 of O111 Tir (corresponding to amino acids 565 to 644 in Figure 5B), followed by the linker Ser-Gly-Ser-Gly (SEQ ID NO: 221), followed by amino acids 243 to 296 of O26 Tir (corresponding to amino acids 651 to 705 in Figure 5B), followed by the linker Ser-Ser-Gly-Gly (SEQ ID NO: 222), followed by amino acids 318 to 347 of O103 (corresponding to amino acids 712 to 741 in Figure 5B). Amino acids 559-564, 645-650 and 706-711 in Figure 5B represent restriction sites used to insert the Tir fragments.

II. Protein Conjugates

In order to enhance immunogenicity of the STEC proteins and multiple epitope fusion molecules, they may be conjugated with a carrier. By "carrier" is meant any molecule which when associated with an antigen of interest, imparts immunogenicity to the antigen. Examples of suitable carriers include large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine,

and the like; amino acid copolymers; inactive virus particles; bacterial toxins such as tetanus toxoid, serum albumins, keyhole limpet hemocyanin, thyroglobulin, ovalbumin, sperm whale myoglobin, and other proteins well known to those skilled in the art. Other suitable carriers for the antigens of the present invention include VP6 polypeptides of
5 rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651.

These carriers may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the
10 N-hydroxysuccinimide ester of 3-(4-dithiopyridyl) propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

STEC proteins and multiple epitope fusion molecules can also be conjugated with a member of the RTX family of toxins (as described further below), such as a *Pasteurella*
15 *haemolytica* leukotoxin (LKT) polypeptide. See, e.g., International Publication No. WO 93/08290, published 29 April 1993, as well as U.S. Patent Nos. 5,238,823, 5,273,889, 5,723,129, 5,837,268, 5,422,110, 5,708,155, 5,969,126, 6,022,960, 6,521,746 and 6,797,272.

Leukotoxin polypeptide carriers are derived from proteins belonging to the family
20 of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (SEQ ID NO: 219) (Highlander et al., *DNA* (1989) 8:15-28), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Lo, *Can. J. Vet. Res.*
25 (1990) 54:S33-S35; Welch, *Mol. Microbiol.* (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35). The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al., *Infect. Immun.*
30 (1985) 50:667-67; Lo et al., *Infect. Immun.* (1987) 55:1987-1996; Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Highlander et al., *DNA* (1989) 8:15-28; Welch, *Mol. Microbiol.* (1991) 5:521-528.

Particular examples of immunogenic leukotoxin polypeptides for use herein include LKT 342, LKT 352, LKT 111, LKT 326 and LKT 101 which are described in greater detail below.

By "LKT 352" is meant a protein which is derived from the *lktA* gene present in plasmid pAA352 (Figure 10) and described in U.S. Patent No. 5,476,657.

LKT 352 has an N-terminal truncation of the native leukotoxin sequence and includes amino acids 38-951 of the native molecule. Thus, the gene in plasmid pAA352 encodes a truncated leukotoxin, having 914 amino acids which lacks the cytotoxic portion of the molecule. The nucleotide and amino acid sequences of LKT 352 is shown in Figures 11A-11I.

By "LKT 111" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pCB111. The plasmid and nucleotide sequence of this gene and the corresponding amino acid sequence are described in U.S. Patent Nos. 5,723,129 and 5,969,126. The gene encodes a shortened version of leukotoxin which was developed from the recombinant leukotoxin gene present in plasmid pAA352 by removal of an internal DNA fragment of approximately 1300 bp in length. The LKT 111 polypeptide has an estimated molecular weight of 52 kDa (as compared to the 99 kDa LKT 352 polypeptide), retains the ability to act as a carrier molecule, and contains convenient restriction sites for use in producing the fusion proteins of the present invention.

By "LKT 101" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pAA101. The plasmid and sequence of LKT 101 is described in U.S. Patent No. 5,476,657 (see Figure 3 therein).

The LKT 101 polypeptide is expressed from a C-terminally truncated form of the *lktA* gene which contains the 5' end of the gene up to the unique *Pst*I restriction endonuclease site. Thus, LKT 101 includes the first 377 amino acids of native, full-length, *P. haemolytica* leukotoxin.

By "LKT 342" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pAA342, described in U.S. Patent No. 5,476,657.

LKT 342 has an N-terminal and C-terminal truncation of the native

leukotoxin sequence and includes amino acids 38-334 of native leukotoxin.

The various LKT molecules described above are representative and other leukotoxin molecules which enhance the immunogenicity of the STEC proteins and fusions will also find use herein. Moreover, the leukotoxin molecules need not be
 5 physically derived from the sequence present in the corresponding plasmids but may be generated in any manner, including for example, by chemical synthesis or recombinant production, as described below.

Additionally, the STEC proteins and multiple epitope fusion molecules can be fused to either the carboxyl or amino terminals or both of the carrier molecule, or at sites
 10 internal to the carrier.

Carriers can be physically conjugated to the proteins of interest, using standard coupling reactions. Alternatively, chimeric molecules can be prepared recombinantly for use in the present invention, such as by fusing a gene encoding a suitable polypeptide carrier to one or more copies of a gene, or fragment thereof, encoding for selected STEC
 15 proteins or STEC multiple epitope fusion molecules.

The nucleotide and amino acid sequences of an exemplary chimeric construct including a leukotoxin carrier is shown in Figures 6A and 6B, respectively and a diagrammatic representation of the sequence is shown in Figures 9C. This construct is identical to the chimeric Tir construct described above, with the exception that a leukotoxin carrier
 20 molecule is present at the N-terminus.

The protein includes in N-terminal to C-terminal order a short vector sequence from pAA352 (corresponding to amino acids 1-9 of Figure 6B), LKT 352 (corresponding to amino acids 10-923 of Figure 6B), a short vector sequence from pAA352 (amino acids 924-926 of Figure 6B), amino acids 2 to 558 of O157 Tir (corresponding to amino acids
 25 927 to 1483 in Figure 6B), followed by the linker Gly-Ser-Gly-Ser (SEQ ID NO: 220), followed by amino acids 279 to 358 of O111 Tir (corresponding to amino acids 1490 to 1569 in Figure 6B), followed by the linker Ser-Gly-Ser-Gly (SEQ ID NO: 221), followed by amino acids 243 to 296 of O26 Tir (corresponding to amino acids 1576 to 1630 in Figure 6B), followed by the linker Ser-Ser-Gly-Gly (SEQ ID NO: 222), followed by amino
 30 acids 318 to 347 of O103 (corresponding to amino

acids 1635 to 1666 in Figure 6B). Amino acids 1484-1489, 1570-1575 and 1631-1634 in Figure 6B represent restriction sites used to insert the Tir fragments.

III. Production of STEC Proteins, Multiple Epitope Fusion Constructs and 5 Conjugates

The STEC proteins and immunogenic fragments thereof, and conjugates with carrier molecules, can be prepared in any suitable manner (e.g. recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g. native, mutant, fusions, etc.). Means for preparing such proteins and conjugates are well
10 understood in the art. Proteins and conjugates are preferably prepared in substantially pure form (i.e. substantially free from other host cell or non host cell proteins).

The proteins and conjugates thereof can be conveniently synthesized chemically, by any of several techniques that are known to those skilled in the peptide art. In general, these methods employ the sequential addition of one or more amino acids to a growing
15 peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected or derivatized amino acid can then be either attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected, under conditions that allow for the formation of an amide linkage. The
20 protecting group is then removed from the newly added amino acid residue and the next amino acid (suitably protected) is then added, and so forth. After the desired amino acids have been linked in the proper sequence, any remaining protecting groups (and any solid support, if solid phase synthesis techniques are used) are removed sequentially or concurrently, to render the final polypeptide. By simple modification of this general
25 procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under conditions which do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis* (Pierce Chemical Co., Rockford, IL 1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, (Academic Press, New
30

York, 1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, (Springer-Verlag, Berlin 1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, Vol. 1, for classical solution synthesis.

5 Typical protecting groups include t-butyloxycarbonyl (Boc),
9-fluorenylmethoxycarbonyl (Fmoc) benzyloxycarbonyl (Cbz); p-toluenesulfonyl (Tx);
2,4-dinitrophenyl; benzyl (Bzl); biphenylisopropylloxycarboxy-carbonyl,
t-amylloxycarbonyl, isobornylloxycarbonyl, o-bromobenzyloxycarbonyl, cyclohexyl,
isopropyl, acetyl, o-nitrophenylsulfonyl and the like. Typical solid supports are
10 cross-linked polymeric supports. These can include divinylbenzene
cross-linked-styrene-based polymers, for example, divinylbenzene-hydroxymethylstyrene
copolymers, divinylbenzene-chloromethylstyrene copolymers and
divinylbenzene-benzhydrylaminopolystyrene copolymers.

 The proteins and conjugates of the present invention can also be chemically
15 prepared by other methods such as by the method of simultaneous multiple peptide
synthesis. See, e.g., Houghten *Proc. Natl. Acad. Sci. USA* (1985) 82:5131-5135; U.S.
Patent No. 4,631,211.

 Alternatively, the above-described proteins and conjugates can be produced
recombinantly. See, e.g., International Publication Nos. WO 97/40063 and WO
20 99/24576, and U.S. Patent No. 7,300,659, for a description of the production of
representative recombinant STEC proteins.

 The proteins of the invention optionally have, but
need not always include, an N-terminal methionine for expression.

 Once coding sequences for the desired proteins have been isolated or synthesized,
25 they can be cloned into any suitable vector or replicon for expression. Numerous cloning
vectors are known to those of skill in the art, and the selection of an appropriate cloning
vector is a matter of choice. A variety of bacterial, yeast, plant, mammalian and insect
expression systems are available in the art and any such expression system can be used.
Optionally, a polynucleotide encoding these proteins can be translated in a cell-free
30 translation system. Such methods are well known in the art.

Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, generally, DNA Cloning: Vols. I & II, *supra*; Sambrook et al., *supra*; B. Perbal, *supra*.

Insect cell expression systems, such as baculovirus systems, can also be used and are known to those of skill in the art and described in, *e.g.*, Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego Calif. ("MaxBac" kit).

Plant expression systems can also be used to produce the immunogenic proteins. Generally, such systems use virus-based vectors to transfect plant cells with heterologous genes. For a description of such systems see, *e.g.*, Porta et al., Mol. Biotech. (1996) 5:209-221; and Hackiand et al., Arch. Virol. (1994) 139:1-22.

Viral systems, such as a vaccinia based infection/transfection system, as described in Tomei et al., J. Virol. (1993) 67:4017-4026 and Selby et al., J. Gen. Virol. (1993) 74:1103-1113, will also find use with the present invention. In this system, cells are first transfected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the DNA of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation product(s).

The coding sequence can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to

herein as "control" elements), so that the DNA sequence encoding the desired immunogenic peptide is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. Leader sequences can be removed by the host in post-translational processing. See, *e.g.*, U.S. Pat. Nos. 4,431,739; 4,425,437; 4,338,397.

Other regulatory sequences may also be desirable which allow for regulation of expression of the peptide sequences relative to the growth of the host cell. Such regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; *i.e.*, to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the immunogenic proteins. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, *e.g.*, Sambrook et al., *supra*; DNA Cloning, Vols. I and II, *supra*; Nucleic Acid Hybridization, *supra*.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus*

spp., will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guilliermondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*.

5 Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the peptides of the present invention are produced by growing host cells transformed by an expression vector
10 described above under conditions whereby the protein of interest is expressed. The selection of the appropriate growth conditions is within the skill of the art. The cells are then disrupted, using chemical, physical or mechanical means, which lyse the cells yet keep the peptides substantially intact. Intracellular proteins can also be obtained by removing components from the cell wall or membrane, e.g., by the use of detergents or
15 organic solvents, such that leakage of the immunogenic polypeptides occurs. Such methods are known to those of skill in the art and are described in, e.g., Protein Purification Applications: A Practical Approach, (E. L. V. Harris and S. Angal, Eds., 1990).

For example, methods of disrupting cells for use with the present invention include
20 but are not limited to: sonication or ultrasonication; agitation; liquid or solid extrusion; heat treatment; freeze-thaw; desiccation; explosive decompression; osmotic shock; treatment with lytic enzymes including proteases such as trypsin, neuraminidase and lysozyme; alkali treatment; and the use of detergents and solvents such as bile salts, sodium dodecylsulphate, TritonTM, NP40 and CHAPSTM. The particular technique used to
25 disrupt the cells is largely a matter of choice and will depend on the cell type in which the polypeptide is expressed, culture conditions and any pre-treatment used.

Following disruption of the cells, cellular debris is removed, generally by centrifugation, and the intracellularly produced protein is further purified, using standard purification techniques such as but not limited to, column chromatography, ion-exchange

chromatography, size-exclusion chromatography, electrophoresis, HPLC, immunoadsorbent techniques, affinity chromatography, immunoprecipitation, and the like.

For example, one method for obtaining the intracellular protein of the present invention involves affinity purification, such as by immunoaffinity chromatography using specific antibodies. The choice of a suitable affinity resin is within the skill in the art. After affinity purification, the peptide can be further purified using conventional techniques well known in the art, such as by any of the techniques described above.

IV. STEC Antibodies

The STEC proteins and multiple epitope fusion proteins of the present invention can be used to produce antibodies for therapeutic, diagnostic and purification purposes. These antibodies may be polyclonal or monoclonal antibody preparations, monospecific antisera, human antibodies, or may be hybrid or chimeric antibodies, such as humanized antibodies, altered antibodies, F(ab')₂ fragments, F(ab) fragments, Fv fragments, single-domain antibodies, dimeric or trimeric antibody fragment constructs, minibodies, or functional fragments thereof which bind to the antigen in question. Antibodies are produced using techniques well known to those of skill in the art and disclosed in, for example, U.S. Patent Nos. 4,011,308; 4,722,890; 4,016,043; 3,876,504; 3,770,380; and 4,372,745.

For example, the proteins can be used to produce STEC-specific polyclonal and monoclonal antibodies for use in diagnostic and detection assays, for purification and for use as therapeutics, such as for passive immunization. Such polyclonal and monoclonal antibodies specifically bind to the STEC proteins in question. In particular, the STEC proteins can be used to produce polyclonal antibodies by administering the proteins to a mammal, such as a mouse, a rat, a rabbit, a goat, or a horse. Serum from the immunized animal is collected and the antibodies are purified from the plasma by, for example, precipitation with ammonium sulfate, followed by chromatography, preferably affinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Mouse and/or rabbit monoclonal antibodies directed against epitopes present in the

cell surface antigen can also be readily produced. In order to produce such monoclonal antibodies, the mammal of interest, such as a rabbit or mouse, is immunized, such as by mixing or emulsifying the antigen in saline, preferably in an adjuvant such as Freund's complete adjuvant ("FCA"), and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). The animal is generally boosted 2-6 weeks later with one or more injections of the antigen in saline, preferably using Freund's incomplete adjuvant ("FIA").

Antibodies may also be generated by *in vitro* immunization, using methods known in the art. See, e.g., James et al., *J. Immunol. Meth.* (1987) 100:5-40.

Polyclonal antisera is then obtained from the immunized animal. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells (splenocytes) may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the antigen. B-cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated splenocytes, are then induced to fuse with cells from an immortalized cell line (also termed a "fusion partner"), to form hybridomas. Typically, the fusion partner includes a property that allows selection of the resulting hybridomas using specific media. For example, fusion partners can be hypoxanthine/aminopterin/thymidine (HAT)-sensitive.

If rabbit-rabbit hybridomas are desired, the immortalized cell line will be from a rabbit. Such rabbit-derived fusion partners are known in the art and include, for example, cells of lymphoid origin, such as cells from a rabbit plasmacytoma as described in Spieker-Polet et al., *Proc. Natl. Acad. Sci. USA* (1995) 92:9348-9352 and U.S. Patent No. 5,675,063, or the TP-3 fusion partner described in U.S. Patent No. 4,859,595.

If a rabbit-mouse hybridoma or a rat-mouse or mouse-mouse hybridoma, or the like, is desired, the mouse fusion partner will be derived from an immortalized cell line from a mouse, such as a cell of lymphoid origin, typically from a mouse myeloma cell line. A number of such cell lines are known in the art and are available from the ATCC.

Fusion is accomplished using techniques well known in the art. Chemicals that promote fusion are commonly referred to as fusogens. These agents are extremely hydrophilic and facilitate membrane contact. One particularly preferred method of cell fusion uses polyethylene glycol (PEG). Another method of cell fusion is electrofusion.

5 In this method, cells are exposed to a predetermined electrical discharge that alters the cell membrane potential. Additional methods for cell fusion include bridged-fusion methods. In this method, the antigen is biotinylated and the fusion partner is avidinylated. When the cells are added together, an antigen-reactive B cell-antigen-biotin-avidin-fusion partner bridge is formed. This permits the specific fusion of an antigen-reactive cell with an
10 immortalizing cell. The method may additionally employ chemical or electrical means to facilitate cell fusion.

Following fusion, the cells are cultured in a selective medium (e.g., HAT medium).

In order to enhance antibody secretion, an agent that has secretory stimulating effects can optionally be used, such as IL-6. See, e.g., Liguori et al., *Hybridoma* (2001) 20:189-198.

15 The resulting hybridomas can be plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (e.g., as ascites in mice). For example, hybridomas producing STEC
20 protein-specific antibodies can be identified using RIA or ELISA and isolated by cloning in semi-solid agar or by limiting dilution. Clones producing the desired antibodies can be isolated by another round of screening.

An alternative technique for generating the monoclonal antibodies of the present invention is the selected lymphocyte antibody method (SLAM). This method involves
25 identifying a single lymphocyte that is producing an antibody with the desired specificity or function within a large population of lymphoid cells. The genetic information that encodes the specificity of the antibody (i.e., the immunoglobulin V_H and V_L DNA) is then rescued and cloned. See, e.g., Babcook et al., *Proc. Natl. Acad. Sci. USA* (1996) 93:7843-7848, for a description of this method.

30 For further descriptions of rabbit monoclonal antibodies and methods of making

the same from rabbit-rabbit and rabbit-mouse fusions, see, e.g., U.S. Patent Nos. 5,675,063 (rabbit-rabbit); 4,859,595 (rabbit-rabbit); 5,472,868 (rabbit-mouse); and 4,977,081 (rabbit-mouse). For a description of the production of conventional mouse monoclonal antibodies, see, e.g., Kohler and Milstein, *Nature* (1975) 256:495-497.

5 It may be desirable to provide chimeric antibodies. By “chimeric antibodies” is intended antibodies that are preferably derived using recombinant techniques and which comprise both human (including immunologically “related” species, e.g., chimpanzee) and non-human components. Such antibodies are also termed “humanized antibodies.” Preferably, humanized antibodies contain minimal sequence derived from non-human
10 immunoglobulin sequences. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. See, for example, U.S. Patent Nos. 5,225,539;
15 5,585,089; 5,693,761; 5,693,762; 5,859,205. In some instances, framework residues of the human immunoglobulin are replaced by corresponding non-human residues (see, for example, U.S. Patents 5,585,089; 5,693,761; 5,693,762). Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance
20 (e.g., to obtain desired affinity). In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at
25 least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones et al., *Nature* (1986) 331:522-525; Riechmann et al., *Nature* (1988) 332:323-329; and Presta, *Curr. Op. Struct. Biol.* (1992) 2:593-596.

Also encompassed are xenogeneic or modified antibodies produced in a
30 non-human mammalian host, more particularly a transgenic mouse, characterized by

inactivated endogenous immunoglobulin (Ig) loci. In such transgenic animals, competent endogenous genes for the expression of light and heavy subunits of host immunoglobulins are rendered non-functional and substituted with the analogous human immunoglobulin loci. These transgenic animals produce human antibodies in the substantial absence of light or heavy host immunoglobulin subunits. See, for example, U.S. Patent No. 5,939,598.

Antibody fragments which retain the ability to recognize the protein of interest, will also find use herein. A number of antibody fragments are known in the art which comprise antigen-binding sites capable of exhibiting immunological binding properties of an intact antibody molecule. For example, functional antibody fragments can be produced by cleaving a constant region, not responsible for antigen binding, from the antibody molecule, using e.g., pepsin, to produce F(ab')₂ fragments. These fragments will contain two antigen binding sites, but lack a portion of the constant region from each of the heavy chains. Similarly, if desired, Fab fragments, comprising a single antigen binding site, can be produced, e.g., by digestion of polyclonal or monoclonal antibodies with papain. Functional fragments, including only the variable regions of the heavy and light chains, can also be produced, using standard techniques such as recombinant production or preferential proteolytic cleavage of immunoglobulin molecules. These fragments are known as FV. See, e.g., Inbar et al., *Proc. Nat. Acad. Sci. USA* (1972) 69:2659-2662; Hochman et al., *Biochem.* (1976) 15:2706-2710; and Ehrlich et al., *Biochem.* (1980) 19:4091-4096.

A phage-display system can be used to expand antibody molecule populations *in vitro*. Saiki, et al., *Nature* (1986) 324:163; Scharf et al., *Science* (1986) 233:1076; U.S. Patent Nos. 4,683,195 and 4,683,202; Yang et al., *J Mol Biol.* (1995) 254:392; Barbas, III et al., *Methods: Comp. Meth Enzymol.* (1995) 8:94; Barbas, III et al., *Proc Natl Acad Sci USA* (1991) 88:7978.

Once generated, the phage display library can be used to improve the immunological binding affinity of the Fab molecules using known techniques. See, e.g., Figini et al., *J. Mol. Biol.* (1994) 239:68. The coding sequences for the heavy and light chain portions of the Fab molecules selected from the phage display library can be isolated

or synthesized, and cloned into any suitable vector or replicon for expression. Any suitable expression system can be used, including those described above.

Single chain antibodies can also be produced. A single-chain Fv ("sFv" or "scFv") polypeptide is a covalently linked VH-VL heterodimer which is expressed from a gene fusion including VH- and VL-encoding genes linked by a peptide-encoding linker. Huston et al., *Proc. Nat. Acad. Sci. USA* (1988) 85:5879-5883. A number of methods have been described to discern and develop chemical structures (linkers) for converting the naturally aggregated, but chemically separated, light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. The sFv molecules may be produced using methods described in the art. See, e.g., Huston et al., *Proc. Nat. Acad. Sci. USA* (1988) 85:5879-5883; U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Design criteria include determining the appropriate length to span the distance between the C-terminus of one chain and the N-terminus of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. See, e.g., U.S. Patent Nos. 5,091,513, 5,132,405 and 4,946,778. Suitable linkers generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility.

"Mini-antibodies" or "minibodies" will also find use with the present invention. Minibodies are sFv polypeptide chains which include oligomerization domains at their C-termini, separated from the sFv by a hinge region. Pack et al., *Biochem.* (1992) 31:1579-1584. The oligomerization domain comprises self-associating α -helices, e.g., leucine zippers, that can be further stabilized by additional disulfide bonds. The oligomerization domain is designed to be compatible with vectorial folding across a membrane, a process thought to facilitate *in vivo* folding of the polypeptide into a functional binding protein. Generally, minibodies are produced using recombinant methods well known in the art. See, e.g., Pack et al., *Biochem.* (1992) 31:1579-1584; Cumber et al., *J. Immunology* (1992) 149B:120-126.

Polynucleotide sequences encoding the antibodies and immunoreactive fragments thereof, described above, are readily obtained using standard techniques, well known in the art, such as those techniques described above with respect to the recombinant production of the STEC proteins.

5 For subjects known to have a STEC disease, an anti-STEC protein antibody may have therapeutic benefit and can be used to confer passive immunity to the subject in question. Alternatively, antibodies can be used in diagnostic applications, described further below, as well as for purification of the STEC proteins.

10 V. Immunogenic Compositions

Once the above proteins, conjugates, antibodies and, if desired, additional recombinant and/or purified proteins are produced, they are formulated into compositions for delivery to a mammalian subject. The active components are typically mixed with a pharmaceutically acceptable vehicle or excipient. Suitable vehicles are, for example,
15 water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants in the case of vaccine compositions, which enhance the effectiveness of the vaccine. Suitable adjuvants are described further below. The compositions of the present invention can also include ancillary substances,
20 such as pharmacological agents, cytokines, or other biological response modifiers.

As explained above, vaccine compositions of the present invention may include adjuvants to further increase the immunogenicity of one or more of the STEC antigens. Such adjuvants include any compound or compounds that act to increase an immune response to a STEC antigen or combination of antigens, thus reducing the quantity of
25 antigen necessary in the vaccine, and/or the frequency of injection necessary in order to generate an adequate immune response. Adjuvants may include for example, emulsifiers, muramyl dipeptides, avridine, aqueous adjuvants such as aluminum hydroxide, chitosan-based adjuvants, and any of the various saponins, oils, and other substances known in the art, such as Amphigen™, LPS, bacterial cell wall extracts, bacterial DNA,
30 synthetic oligonucleotides and combinations thereof (Schijns et al., *Curr. Opi. Immunol.*

(2000) 12:456), *Mycobacterium phlei* (*M. phlei*) cell wall extract (MCWE) (U.S. Patent No. 4,744,984), *M. phlei* DNA (M-DNA), M-DNA-*M. phlei* cell wall complex (MCC). For example, compounds which may serve as emulsifiers herein include natural and synthetic emulsifying agents, as well as anionic, cationic and nonionic compounds. Among the synthetic compounds, anionic emulsifying agents include, for example, the potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids (i.e., metallic soaps), and organic sulfonates such as sodium lauryl sulfate. Synthetic cationic agents include, for example, cetyltrimethylammonium bromide, while synthetic nonionic agents are exemplified by glyceryl esters (e.g., glyceryl monostearate), polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters (e.g., sorbitan monopalmitate) and their polyoxyethylene derivatives (e.g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol.

Other suitable adjuvants can be formed with an oil component, such as a single oil, a mixture of oils, a water-in-oil emulsion, or an oil-in-water emulsion. The oil may be a mineral oil, a vegetable oil, or an animal oil. Mineral oil, or oil-in-water emulsions in which the oil component is mineral oil are preferred. In this regard, a "mineral oil" is defined herein as a mixture of liquid hydrocarbons obtained from petrolatum via a distillation technique; the term is synonymous with "liquid paraffin," "liquid petrolatum" and "white mineral oil." The term is also intended to include "light mineral oil," i.e., an oil which is similarly obtained by distillation of petrolatum, but which has a slightly lower specific gravity than white mineral oil. See, e.g., *Remington's Pharmaceutical Sciences*, *supra*. A particularly preferred oil component is the oil-in-water emulsion sold under the trade name of EMULSIGEN PLUS™ (comprising a light mineral oil as well as 0.05% formalin, and 30 mcg/mL gentamicin as preservatives), available from MVP Laboratories, Ralston, Nebraska. Another preferred adjuvant for use herein is an adjuvant known as "VSA3" which is a modified form of the EMULSIGEN PLUS™ adjuvant which includes DDA (see, U.S. Patent No. 5,951,988).

Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil, all of which are available commercially. Suitable vegetable

oils, include, without limitation, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, soybean oil, and the like.

Alternatively, a number of aliphatic nitrogenous bases can be used as adjuvants with the vaccine formulations. For example, known immunologic adjuvants include
5 amines, quaternary ammonium compounds, guanidines, benzamidines and thiouroniums (Gall, D. (1966) *Immunology* 11:369-386). Specific compounds include dimethyldioctadecylammonium bromide (DDA) (available from Kodak) and N,N-dioctadecyl-N,N-bis(2-hydroxyethyl)propanediamine ("avridine"). The use of DDA as an immunologic adjuvant has been described; see, e.g., the Kodak Laboratory
10 Chemicals Bulletin 56(1):1-5 (1986); *Adv. Drug Deliv. Rev.* 5(3):163-187 (1990); *J. Controlled Release* 7:123-132 (1988); *Clin. Exp. Immunol.* 78(2):256-262 (1989); *J. Immunol. Methods* 97(2):159-164 (1987); *Immunology* 58(2):245-250 (1986); and *Int. Arch. Allergy Appl. Immunol.* 68(3):201-208 (1982). Avridine is also a well-known adjuvant. See, e.g., U.S. Patent No. 4,310,550 to Wolff, III et al., which describes the use
15 of N,N-higher alkyl-N',N'-bis(2-hydroxyethyl)propane diamines in general, and avridine in particular, as vaccine adjuvants. U.S. Patent No. 5,151,267 to Babiuk, and Babiuk et al. (1986) *Virology* 159:57-66, also relate to the use of avridine as a vaccine adjuvant.

The vaccine compositions can be prepared by uniformly and intimately bringing into association the STEC protein preparations and the adjuvant using techniques well
20 known to those skilled in the art including, but not limited to, mixing, sonication and microfluidation. The adjuvant will preferably comprise about 10 to 50% (v/v) of the vaccine, more preferably about 20 to 40% (v/v) and most preferably about 20 to 30% or 35% (v/v), or any integer within these ranges.

The compositions of the present invention are normally prepared as injectables,
25 either as liquid solutions or suspensions, or as solid forms which are suitable for solution or suspension in liquid vehicles prior to injection. The preparation may also be prepared in solid form, emulsified or the active ingredient encapsulated in liposome vehicles or other particulate carriers used for sustained delivery. For example, the vaccine may be in the form of an oil emulsion, water in oil emulsion, water-in-oil-in-water emulsion,
30 site-specific emulsion, long-residence emulsion, sticky-emulsion, microemulsion,

nanoemulsion, liposome, microparticle, microsphere, nanosphere, nanoparticle and various natural or synthetic polymers, such as nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and HytrelTM 7 copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters
5 such as those used to make resorbable sutures, that allow for sustained release of the vaccine.

Furthermore, the polypeptides may be formulated into compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with
10 inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

15 Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The composition is formulated to contain an effective amount of the desired STEC protein or multiple epitope fusion, the exact amount being readily determined by one
20 skilled in the art, wherein the amount depends on the animal to be treated and the capacity of the animal's immune system to synthesize antibodies. The composition or formulation to be administered will contain a quantity of one or more of the STEC antigens described herein adequate to achieve the desired state in the subject being treated. For purposes of the present invention, a therapeutically effective amount of a vaccine comprising the STEC
25 proteins with or without added recombinant and/or purified STEC antigens, contains about 0.05 to 1500 µg of the STEC protein, preferably about 10 to 1000 µg of the protein, more preferably about 30 to 500 µg and most preferably about 40 to 300 µg, such as 50 to 200 µg, or any integer between these values. Routes of administration include, but are not limited to, oral, topical, subcutaneous, intramuscular, intravenous, subcutaneous,
30 intradermal, transdermal and subdermal. Depending on the route of administration, the

volume per dose is preferably about 0.001 to 10 ml, more preferably about 0.01 to 5 ml, and most preferably about 0.1 to 3 ml. Vaccine can be administered in a single dose treatment or in multiple dose treatments (boosts) on a schedule and over a time period appropriate to the age, weight and condition of the subject, the particular vaccine
5 formulation used, and the route of administration.

Any suitable pharmaceutical delivery means may be employed to deliver the compositions to the vertebrate subject. For example, conventional needle syringes, spring or compressed gas (air) injectors (U.S. Patent Nos. 1,605,763 to Smoot; 3,788,315 to Laurens; 3,853,125 to Clark et al.; 4,596,556 to Morrow et al.; and 5,062,830 to Dunlap),
10 liquid jet injectors (U.S. Patent Nos. 2,754,818 to Scherer; 3,330,276 to Gordon; and 4,518,385 to Lindmayer et al.), and particle injectors (U.S. Patent Nos. 5,149,655 to McCabe et al. and 5,204,253 to Sanford et al.) are all appropriate for delivery of the compositions.

If a jet injector is used, a single jet of the liquid vaccine composition is ejected
15 under high pressure and velocity, e.g., 1200-1400 PSI, thereby creating an opening in the skin and penetrating to depths suitable for immunization.

VI. Nucleic Acid-based Immunization Methods

Generally, nucleic acid-based vaccines for use with the present invention will
20 include relevant regions encoding the desired STEC protein or fusion, with suitable control sequences and, optionally, ancillary therapeutic nucleotide sequences. The nucleic acid molecules are prepared in the form of vectors which include the necessary elements to direct transcription and translation in a recipient cell, as described above.

In order to augment an immune response in an immunized subject, the nucleic acid
25 molecules can be administered in conjunction with ancillary substances, such as pharmacological agents, adjuvants, or in conjunction with delivery of vectors encoding biological response modifiers such as cytokines and the like.

Once prepared, the nucleic acid vaccine compositions can be delivered to the subject using known methods. In this regard, various techniques for immunization with
30 antigen-encoding DNAs have been described. See, e.g., U.S. Patent No. 5,589,466 to

Felgner et al.; Tang et al. (1992) *Nature* 358:152; Davis et al. (1993) *Hum. Molec. Genet.* 2:1847; Ulmer et al. (1993) *Science* 258:1745; Wang et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:4156; Eisenbraun et al. (1993) *DNA Cell Biol.* 12:791; Fynan et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:12476; Fuller et al. (1994) *AIDS Res. Human Retrovir.* 10:1433; and Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519. General methods for delivering nucleic acid molecules to cells *in vitro*, for the subsequent reintroduction into the host, can also be used, such as liposome-mediated gene transfer. See, e.g., Hazinski et al. (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278-281; Canonico et al. (1991) *Clin. Res.* 39:219A; and Nabel et al. (1990) *Science* 249:1285-1288. Thus, the nucleic acid vaccine compositions can be delivered in either liquid or particulate form using a variety of known techniques. Typical vaccine compositions are described above.

VII. Tests to Determine the Efficacy of an Immune Response

One way of assessing efficacy of therapeutic treatment and prevention involves monitoring immune responses against the STEC proteins and fusions in the compositions of the invention after administration of the composition. Another way of assessing efficacy involves monitoring infection after administration of a composition of the invention. Moreover, efficacy of the compositions can be determined by assessing whether a reduction of the amount of STEC in the intestinal tract in the subject is achieved, thus reducing transmission of disease by reducing the amount of fecal shedding of bacteria, and/or the time period of STEC shedding by an animal is reduced.

Another way of assessing the immunogenicity of the proteins of the immunogenic compositions of the present invention is to express the proteins recombinantly and to screen the subject's sera by immunoblot. A positive reaction between the protein and the serum indicates that the subject has previously mounted an immune response to the protein in question and thus the protein is an immunogen. This method may also be used to identify immunodominant proteins and/or epitopes.

Another way of checking efficacy involves monitoring infection after administration of the compositions of the invention. One way of checking efficacy

involves monitoring immune responses both systemically (such as monitoring the level of IgG1 and IgG2a production) and mucosally (such as monitoring the level of IgA production) against the antigens in the compositions of the invention after administration of the composition. Typically, serum-specific antibody responses are determined
5 post-immunization but pre-challenge whereas mucosal specific antibody body responses are determined post-immunization and post-challenge.

The immunogenic compositions of the present invention can be evaluated in *in vitro* and *in vivo* animal models prior to host administration.

The efficacy of immunogenic compositions of the invention can also be determined
10 *in vivo* by challenging animal models of infection with the immunogenic compositions. The immunogenic compositions may or may not be derived from the same strains as the challenge strains. Preferably the immunogenic compositions are derivable from the same strains as the challenge strains.

The immune response may be one or both of a TH1 immune response and a TH2
15 response. The immune response may be an improved or an enhanced or an altered immune response. The immune response may be one or both of a systemic and a mucosal immune response. Preferably the immune response is an enhanced systemic and/or mucosal response.

An enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1
20 and/or TH2 immune response. Preferably, the enhanced immune response includes an increase in the production of IgG1 and/or IgG2a and/or IgA. Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

Activated TH2 cells enhance antibody production and are therefore of value in
25 responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10),

or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune response will include an increase in IgG1 production.

A TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN γ , and TNF β), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

The immunogenic compositions of the invention will preferably induce long lasting (e.g., neutralizing) antibodies and a cell mediated immunity that can quickly respond upon exposure to one or more infectious antigens. By way of example, evidence of neutralizing antibodies in blood samples from the subject is considered as a surrogate parameter for protection.

VIII. Diagnostic Assays

As explained above, the STEC protein, variants, immunogenic fragments and fusions thereof, may also be used as diagnostics to detect the presence of reactive antibodies of STEC, in a biological sample in order to determine the presence of infection.

For example, the presence of antibodies reactive with a STEC protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound antibody in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets

or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. Typically, a solid support is first reacted with a solid phase component (e.g., one or more STEC proteins or fusions) under suitable binding conditions such that the component is sufficiently immobilized to the support. Sometimes, immobilization of the antigen to the support can be enhanced by first coupling the antigen to a protein with better binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other molecules that can be used to bind the antigens to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to the antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. *Bioconjugate Chem.* (1992) 3:2-13; Hashida et al., *J. Appl. Biochem.* (1984) 6:56-63; and Anjaneyulu and Staros, *International J. of Peptide and Protein Res.* (1987) 30:117-124.

After reacting the solid support with the solid phase component, any non-immobilized solid-phase components are removed from the support by washing, and the support-bound component is then contacted with a biological sample suspected of containing ligand moieties (e.g., antibodies toward the immobilized antigens) under suitable binding conditions. After washing to remove any non-bound ligand, a secondary binder moiety is added under suitable binding conditions, wherein the secondary binder is capable of associating selectively with the bound ligand. The presence of the secondary binder can then be detected using techniques well known in the art.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a STEC protein or fusion. A biological sample containing or suspected of containing anti- *S. Enteritidis* immunoglobulin molecules is then added to the coated wells. After a period of incubation sufficient to allow antibody binding to the immobilized antigen, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is

allowed to react with any captured sample antibodies, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Thus, in one particular embodiment, the presence of bound anti-STE_C ligands from a biological sample can be readily detected using a secondary binder comprising an antibody directed against the antibody ligands. A number of immunoglobulin (Ig) molecules are known in the art which can be readily conjugated to a detectable enzyme label, such as horseradish peroxidase, alkaline phosphatase or urease, using methods known to those of skill in the art. An appropriate enzyme substrate is then used to generate a detectable signal. In other related embodiments, competitive-type ELISA techniques can be practiced using methods known to those skilled in the art.

Assays can also be conducted in solution, such that the STE_C proteins and antibodies specific for those proteins form complexes under precipitating conditions. In one particular embodiment, STE_C proteins can be attached to a solid phase particle (e.g., an agarose bead or the like) using coupling techniques known in the art, such as by direct chemical or indirect coupling. The antigen-coated particle is then contacted under suitable binding conditions with a biological sample suspected of containing antibodies for the STE_C proteins. Cross-linking between bound antibodies causes the formation of particle-antigen-antibody complex aggregates which can be precipitated and separated from the sample using washing and/or centrifugation. The reaction mixture can be analyzed to determine the presence or absence of antibody-antigen complexes using any of a number of standard methods, such as those immunodiagnostic methods described above.

In yet a further embodiment, an immunoaffinity matrix can be provided, wherein a polyclonal population of antibodies from a biological sample suspected of containing anti-STE_C molecules is immobilized to a substrate. In this regard, an initial affinity purification of the sample can be carried out using immobilized antigens. The resultant sample preparation will thus only contain anti-STE_C moieties, avoiding potential nonspecific binding properties in the affinity support. A number of methods of immobilizing immunoglobulins (either intact or in specific fragments) at high yield and good retention of antigen binding activity are known in the art. Not being limited by any

particular method, immobilized protein A or protein G can be used to immobilize immunoglobulins.

Accordingly, once the immunoglobulin molecules have been immobilized to provide an immunoaffinity matrix, labeled STEC proteins are contacted with the bound
5 antibodies under suitable binding conditions. After any non-specifically bound antigen has been washed from the immunoaffinity support, the presence of bound antigen can be determined by assaying for label using methods known in the art.

Additionally, antibodies raised to the STEC proteins, rather than the proteins themselves, can be used in the above-described assays in order to detect the presence of
10 antibodies to the proteins in a given sample. These assays are performed essentially as described above and are well known to those of skill in the art.

IX. Kits

The invention also provides kits comprising one or more containers of
15 compositions of the invention. Compositions can be in liquid form or can be lyophilized, as can individual antigens. Suitable containers for the compositions include, for example, bottles, vials, syringes, and test tubes. Containers can be formed from a variety of materials, including glass or plastic. A container may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper
20 pierceable by a hypodermic injection needle).

The kit can further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It can also contain other materials useful to the end-user, including other pharmaceutically acceptable formulating solutions such as buffers, diluents, filters,
25 needles, and syringes or other delivery device. The kit may further include a third component comprising an adjuvant.

The kit can also comprise a package insert containing written instructions for methods of inducing immunity or for treating infections. The package insert can be an unapproved draft package insert or can be a package insert approved by the Food and Drug
30 Administration (FDA) or other regulatory body.

The invention also provides a delivery device pre-filled with the immunogenic compositions of the invention.

Similarly, antibodies can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can
5 also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

C. Experimental

10 Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course,
15 be allowed for.

Example 1

Construction and Identification of TIR Epitopes

In order to identify Tir epitopes, twenty-two 30-mer peptides with five amino acid
20 overlaps for the STEC O157:H7 Tir protein were constructed (see Table 1). Rabbit polyclonal antisera was raised against TTSPs from STEC O157:H7 and non-O157 TTSPs (O26:H11, O103:H2 and O111:NM) and tested at a dilution of 1/20 against the twenty-two O157:H7 Tir peptides. As shown in Figure 7, very few peptides were recognized by the non-O157 sera. Anti-O103:H2 was the only sera that recognized multiple peptides.

25 In order to construct a chimeric Tir protein, epitopes were identified in the Tir protein from non-O157 STEC serotypes which had diverged from STEC O157:H7, but that were still recognized by the host immune system. Of particular interest was the portion of Tir that spanned amino acids 259 to 363. These amino acids have been shown to be exposed on the surface of the host's epithelial cells, making them a prime target for
30 vaccine development. In total seven 30-mer peptides were constructed for each of the

non-O157 EHEC serotypes (O26:H11, O103:H2 and O111:NM) (Table 1). The cross-reactivity of STEC polyclonal antibodies against TTSPs from the various serotypes was tested as described above.

The non-O157 and the O157:H7 TTSPs polyclonal antibody against the non-O157 peptides showed a similar pattern to that seen with the STEC O157:H7 peptides. The homologous sera showed the best results (Figures 8A-8D). Peptide number three from the various serotypes displayed the most reactivity against the non-O157 sera. These results demonstrate the variability which is found within the Tir protein in STEC serotypes. However, a number of peptides were recognized by the homologous sera which no other serotype recognized.

Table 1

O157	SEQ ID NO	PEPTIDE
1-MPIGNLGHNPVNNNSIPPAPPLPSQTDGAG	1	Tir O157 AA 1-30
2-TDGAGGRGQLINSTGPLGSRALFTPVRNSM	2	Tir O157 AA 26-55
3-VRNSMADSGDNRASDVPLPVNPMRLAASE	3	Tir O157 AA 51-80
4-LAASEITLNDGFEVLHDHGPLDTLNRQIGS	4	Tir O157 AA 76-105
5-RQIGSSVFRVETQEDGKHIAVGQRNGVETS	5	Tir O157 AA 101-130
6-GVETSVVLSDQEYARLQSIDPEGKDKFVFT	6	Tir O157 AA 126-155
7-KFVFTGGRGGAGHAMVTVASDITEARQRIL	7	Tir O157 AA 151-180
8-RQRILELLEPKGTGESKGAGESKGVGELRE	8	Tir O157 AA 176-205
9-GELRESNSGAENTTETQTSTSTSSLRSDPK	9	Tir O157 AA 201-230
10-RSDPKLWLALGT VATGLIGLAATGIVQALA	10	Tir O157 AA 226-255
11-VQALALTPEPDSPTTTDPDAAASATETATR	11	Tir O157 AA 251-280
12-ETATRDQLTKEAFQNP DNQKVNIDELGNAI	12	Tir O157 AA 276-305
13-LGNAIPSGVLKDDVVANIEEQAKAAGEEAK	13	Tir O157 AA 301-330
14-GEEAKQQAIENNAQAQKKYDEQQAKRQEEL	14	Tir O157 AA 326-355
15-RQEELKVSSGAGYGLSGALILGGGIGVAVT	15	Tir O157 AA 351-380
16-GVAVTAALHRKNQPVEQTTTTTTTTTTTSA	16	Tir O157 AA 376-405
17-TTTSARTVENK PANNTPAQGNVDTPGSED	17	Tir O157 AA 401-430
18-GSED TMESRRSSMASTSTFFDTSSIGTVQ	18	Tir O157 AA 426-455
19-IGTVQNPYADVKTSLHDSQVPTSNSNTSVQ	19	Tir O157 AA 451-480
20-NTSVQNMGNTDSVVYSTIQHPPRDTTDNGA	20	Tir O157 AA 476-505
21-TDNGARLLGNPSAGIQSTYARLALSGGLRH	21	Tir O157 AA 501-530
22-GLRHDMGGLTGGSNSAVNTSNNPPAPGSHRFV	22	Tir O157 AA 526-558

O26		
1-RADPKLWLSLGTIAAGLIGMAATGIAQAVA	23	Tir O26 AA 218-247
2-AQAVALTPEPDDPITTPDAAANTAEAAAK	24	Tir O26 AA 243-272
3-EAAAKDQLTKEAFQNP DNQKVNIDENGNAI	25	Tir O26 AA 268-297
4-NGNAIPSGELKDDVVAQIAEQAKAAGEQAR	26	Tir O26 AA 293-322
5-GEQARQEAIESNSQAQKKYDEQHAKREQEM	27	Tir O26 AA 318-347
6-REQEMSLSSGVGYGISGALILGGGIGAGVT	28	Tir O26 AA 343-372
7-GAGVTAALHRKNQPAEQTITTRTVVDNQPT	29	Tir O26 AA 368-397
O103		
1-RADPKLWLSLGTIAAGLIGMAATGIAQAVA	30	Tir O103 AA 218-247
2-AQAVALTPEPDDPTTTDPDTAASTAEAAATK	31	Tir O103 AA 243-272
3-EAATKDRLTQEAFQDPDKQKVNIDENGNAI	32	Tir O103 AA 268-297
4-NGNAIPSGELIDDVVAQIAEQAKAAGEQAR	33	Tir O103 AA 293-322
5-GEQARQEAIESNSQAQKKYDEQHAKREQEM	34	Tir O103 AA 318-347
5-GEQARQEAIESNSQAQKKYDEQHAKREQEM	35	Tir O103 AA 343-372
7-GAGVTAALHRKNQPAEQTITTRTVVDNQPT	36	Tir O103 AA 368-397
O111		
1-RSDPKFWVSIGAI AAGLAGLAATGITQALA	37	Tir O111 AA 229-258
2-TQALALTPEPDDPTTTDPEQAASAAESATR	38	Tir O111 AA 254-283
3-ESATRDQLTQEAFKNPENQKVSIDEIGNSI	39	Tir O111 AA 279-308
4-IGNSIPSGELKDDVVAKIEEQAKEAGEAAR	40	Tir O111 AA 304-333
5-GEAARQQAVESNAQAQQR YDTQYARRQEEL	41	Tir O111 AA 329-358
6-RQEELELSSGIGYSLSSALIVGGGIGAGVT	42	Tir O111 AA 354-383
7-GAGVTTALHRRNQPAEQTTTTTHTTVVQQQ	43	Tir O111 AA 379-408

Table 1. Sequence of constructed STEC O157:H7 Tir and non-O157 Tir peptides.
Underlined peptides in O157 section represent the intimin binding domain.

5

Example 2

Construction of Chimeric TIR Proteins

Out of the peptides tested in Example 1, six unique non-O157 30-mer peptides, specific to each serotype were chosen. See, Table 2.

	<i>E. coli</i> non-O157 peptide targets		
	Serotypes		
Peptides	O103:H2	O26:H11	O111:N M
1			
2		X	
3		X	X
4			X
5	X		X
6			
7			

X=selected peptides

5 **Table 2. Targets selected to be fused with STEC O157:H7 Tir protein**

DNA encoding these non-O157 peptides was linked to the 3' end of DNA encoding the STEC O157:H7 Tir protein. Primers and restriction sites are shown in Table 3. Each peptide was designed to be separated by four amino acids selected from Gly and Ser to improve flexibility of the protein (See, Figures 9A and 9B). The nucleotide sequence and amino acid sequence of the chimeric Tir protein is shown in Figures 5A and 5B, respectively (SEQ ID NOS:51 and 52). The protein includes in N-terminal to C-terminal order the full-length O157 Tir sequence (amino acids 1 to 558 of Figure 5B), followed by the linker Gly-Ser-Gly-Ser, followed by amino acids 279 to 358 of O111 Tir (corresponding to amino acids 565 to 644 in Figure 5B), followed by the linker Ser-Gly-Ser-Gly, followed by amino acids 243 to 297 of O26 Tir (corresponding to amino acids 651 to 705 in Figure 5B), followed by the linker Ser-Ser-Gly-Gly, followed by amino acids 318 to 347 of O103 (corresponding to amino acids 712 to 741 in Figure 5B). Amino acids 559-564, 645-650 and 706-711 in Figure 5B represent restriction sites used to insert the Tir fragments.

(1) **TirO157-PEP-F** kpnI
CGGGGTACCCCTATTGGTAATCTTGGTCATAATCCCAATGTGAATAATTC
(SEQ ID NO: 189)

5 **TirO157-PEP-F** GSGS-AgeI-PstI
AAAACTGCAGACCGGTGGAGCCAGAACCGACGAAACGATGGGATCCCG
(SEQ ID NO: 190)

(2) **TirO111-PEP-F** AgeI
GGCTACCGGTGAAAGTGCACAAAGAGATCAGTTAACGCAAGAAGCATTCAAG
(SEQ ID NO: 191)

10 **TirO111-PEP-R** SGSG-SpeI-GS-HindIII
CCCAAGCTTAGAACCACCTAGTCCCCGATCCTGATAATTCCTCCTGACGTCTGGCATAAC
(SEQ ID NO: 192)

(3) **TirO26-PEP-F** SpeI
GGACTAGTGCACAGGCTGTTGCGTTGACTCCAGAGCCGGATG
15 (SEQ ID NO: 193)

TirO26-PEP-R SSGG-NsiI
CCAATGCATTCCGCCGGATGAAATTGCATTTCCGTTCTCATCG
(SEQ ID NO: 194)

(4) **TirO103-PEP-F** NsiI
20 CCAATGCATGGGGAACAGGCCAGACAGGAAG
(SEQ ID NO: 195)

Tir103-PEP-R HindIII
CCCAAGCTTCATTTCTGTTCCGCGTTTAGC
25 (SEQ ID NO: 196)

Table 3. Oligonucleotide primers used for the amplification of STEC Tir and non-O157 Tir peptides. Nucleotide sequence is 5' to 3'

30 These peptides were also used to construct a second chimeric protein which was identical to the first except that it was fused to the leukotoxin carrier LKT 352 (Figure 9C). To do so, the chimeric Tir construct described above was ligated into the plasmid pAA352 as described in U.S. Patent Nos. 5,476,657; 5,422,110; 5,723,129 and 5,837,268.

Plasmid pAA352 is depicted in Figure 10 and expresses LKT 352, the sequence of which is depicted in Figure 11. LKT 352 is derived from the *lktA* gene of *Pasteurella haemolytica* leukotoxin and is a truncated leukotoxin molecule, having 914 amino acids and an estimated molecular weight of around 99 kDa, which lacks the cytotoxic portion of the molecule. The chimeric Tir fusion protein was expressed as a C-terminal fusion of the Lkt protein.

40 The nucleotide sequence and amino acid sequence of the LKT 352/chimeric Tir fusion protein are shown in Figures 6A and 6B (SEQ ID NOS:53 and 54). The protein includes in N-terminal to C-terminal order a short vector sequence from pAA352

(corresponding to amino acids 1-9 of Figure 6B), LKT 352 (corresponding to amino acids 10-923 of Figure 6B), a short vector sequence from pAA352 (amino acids 924-926 of Figure 6B), amino acids 2 to 558 of O157 Tir (corresponding to amino acids 927 to 1483 in Figure 6B), followed by the linker Gly-Ser-Gly-Ser, followed by amino acids 279 to 358 of O111 Tir (corresponding to amino acids 1490 to 1569 in Figure 6B), followed by the linker Ser-Gly-Ser-Gly, followed by amino acids 243 to 297 of O26 Tir (corresponding to amino acids 1576 to 1630 in Figure 6B), followed by the linker Ser-Ser-Gly-Gly, followed by amino acids 318 to 347 of O103 (corresponding to amino acids 1635 to 1666 in Figure 6B). Amino acids 1484-1489, 1570-1575 and 1631-1634 in Figure 6B represent restriction sites used to insert the Tir fragments.

Both proteins were purified, run on a 12% SDS-PAGE Coomassie-stained gel and used in a Western blot against a STEC O157:H7 anti-Tir monoclonal antibody to confirm that the proper protein was purified.

15

Example 3

Immunogenicity of Chimeric TIR Proteins

In order to test the immunogenicity of the chimeric TIR proteins and to determine whether seroconversion would occur in response to the proteins, separate groups of rabbits were vaccinated with (1) the chimeric Tir construct, (2) the LKT 352/chimeric Tir fusion, (3) O26 Peptide #2 from Table 2, (4) O26 Peptide #3 from Table 2, (5) O103 Peptide #5 from Table 2, (6) O111 Peptide #3 from Table 2, (7) O111 Peptide #4 from Table 2, (8) O111 Peptide #5 from Table 2, (9) the Tir protein from STEC O157:H7 and (10) Peptide SN11 as a negative control. Rabbits were boosted three times (Day 21, Day 42 and Day 57). The vaccine included 50 micrograms of each protein in a formulation that included 30% EMULSIGEN D (MVP Laboratories, Ralston, NE) as an adjuvant.

Two weeks after the final boost, the animals were bled and sera was used in ELISAs to determine seroconversion. As can be seen in Figures 12A-12J, rabbits responded well to the whole chimeric proteins and were also able to respond to the individual non-O157 peptides. It appears that the rabbits responded better to O111 Peptide #5 and O103 Peptide #5 on the chimeric Tir protein than the LKT 352/Tir fusion.

Example 4

Cloning, Expression and Purification of STEC O157:H7 Secreted Proteins

Using an *in vitro* inhibition attachment assay, it was shown that anti-O157:H7
5 TTSPs polyclonal antibody was able to inhibit STEC O157:H7 from attaching to HEp-2
epithelial cells. However, when anti-Tir O157:H7 polyclonal antibody or concentrations of
purified Tir protein were tested, neither was capable of blocking attachment of STEC
O157:H7 to HEp-2 cells. Anti-EspA O157:H7 polyclonal antibody was also tested and
produced the same results as the anti-Tir O157:H7 polyclonal antibody.

10 These results show that there is something present in the anti-O157:H7 TTSPs
polyclonal antibody which is able to inhibit colonization. Tir and EspA which react with
anti-O157:H7 TTSPs polyclonal antibody on a Western blot, were not capable of
inhibiting colonization of STEC O157:H7 to HEp-2 cells when anti-Tir O157:H7
polyclonal antibody and anti-EspA O157:H7 polyclonal antibody was tested. Without
15 being bound to a particular theory, the inhibition of colonization by the anti-O157:H7
TTSPs polyclonal antibody may be due to either a combination or an unidentified protein
secreted into the media.

The STEC O157:H7 TTSPs used to raise the antibody was a cocktail of mostly
unidentified proteins secreted into M9 minimal media. Initially, it was believed that the
20 majority of secreted proteins came from the locus for enterocyte effacement (LEE)
Pathogenicity Island. However, recently several proteins called non-LEE effectors
(NLEs) have been identified which are secreted through the TTSS but are not located on
the LEE Island. Tobe et al., *Proc. Natl. Acad. Sci. USA* (2006) 103:14941-14946 reported
that 39 non-LEE effectors were secreted through the TTSS.

25 40 proteins from genes found on the LEE Pathogenicity Island (excluding intimin),
as well as 29 non-LEE effectors were over-expressed and purified in order to test the
proteins in ELISAs and western blots against anti-O157:H7 TTSPs polyclonal antibody
and anti non-O157 TTSPs polyclonal antibodies.

In particular, all 69 genes were cloned and sequenced using the Qiagen pQE-30
30 HIS-tagged vector cloning system (primers and restrictions sites found on Table 4).

Ni-NTA agarose was used for purification of 6xHis-tagged proteins by gravity-flow chromatography. 66 of these proteins were purified. The remaining three are membrane proteins which have been difficult to purify. These three proteins are members of the inner membrane complex of the secretion apparatus. However, these proteins may not be relevant to the identification of secreted immunogenic proteins based on their location and role.

LEE genes			
ler	F	CGCGGATCCCGGAGATTATTTATTATGAATATGGAAAATAATTCAC (SEQ ID NO:57)	BamHI
	R	CCCAAGCTTTTAAATATTTTTCAGCGGTATTATTTCTTCTTCAGTGTCC (SEQ ID NO:58)	HindIII
orf2	F	CGCGGATCCATAACGATAACTGAGCTGGAAGATG (SEQ ID NO:59)	BamHI
	R	CCCAAGCTTCTATTTATTATTAATCCTGATTCGC (SEQ ID NO:60)	HindIII
cesA/B	F	CGCGGATCCAGTATTGTGAGCCAAACAAGAAATAAAG (SEQ ID NO:61)	BamHI
	R	CCCAAGCTTTCATACTATTTTCTATTATTTCTATTCCG (SEQ ID NO:62)	HindIII
orf4	F	CGCGGATCCACAATTTTAAATAAAATAGAC (SEQ ID NO:63)	BamHI
	R	CCCAAGCTTTCATAAAGTTTCATAAGGC (SEQ ID NO:64)	HindIII
orf5	F	CGCGGATCCCTTACAGAAGATATCATACCAGAGG (SEQ ID NO:65)	BamHI
	R	CCCAAGCTTTCATTCTGAATAATGCTAAG (SEQ ID NO:66)	HindIII
escS*	F	CGCGGATCCCC GTTATCGGTATTATTATTAGTCTGG (SEQ ID NO:67)	BamHI
	R	ACGCGTCGACTTAGCCGTTACCTTCGGAATC (SEQ ID NO:68)	Sall
escT	F	CGCGGATCCAATGAGATAATGACGGTCATAGTATC (SEQ ID NO:69)	BamHI
	R	CCCAAGCTTTCATCTCATTAATCATGCTCGGTAAC (SEQ ID NO:70)	HindIII
rorf13	F	CGCGGATCCAAAAAATAATACTGAGCATCATTCTC (SEQ ID NO:71)	BamHI
	R	CGCGGATCCAAAAAATAATACTGAGCATCATTCTC (SEQ ID NO:72)	HindIII
grlR	F	CGCGGATCCATTATGAAGGATGGCATCTATAGC (SEQ ID NO:73)	BamHI
	R	CCCAAGCTTTTATTTTAAATAAACTTGTGGCATTCTGTG (SEQ ID NO:74)	HindIII
grlA	F	CGCGGATCCGAATCTAAAAATAAAAATGGCGAC (SEQ ID NO:75)	BamHI
	R	CGCGGATCCGAATCTAAAAATAAAAATGGCGAC (SEQ ID NO:76)	HindIII
cesD	F	CGCGGATCCAGCAGGAAATTTAGCTCTCTAG (SEQ ID NO:77)	BamHI
	R	CCCAAGCTTTTACTCTGTATTACCTAAC (SEQ ID NO:78)	HindIII
escC	F	CGCGGATCCAAAAAATAAGTTTTTTTATTTTACAGCACTATTT TGCTGCAGTGCACAAGCTGCCCC (SEQ ID NO:79)	BamHI
	R	CCCAAGCTTTTATTCGCTAGATGCAGATTTTATCGGGGTTGCTTT AATTAAAAAGAGTCGAACAAC (SEQ ID NO:80)	HindIII
sepD	F	CGCGGATCCAACAATAATAATGGCATAGCAAAGAATG (SEQ ID NO:81)	BamHI
	R	CCCAAGCTTTTACACAATTCGTCCTATATCAGAAAAC (SEQ ID NO:82)	HindIII
escJ	F	CGCGGATCCAAAAACACATTAAAAACCTTTTTTTATTGGCTGC (SEQ ID NO:83)	BamHI
	R	CCCAAGCTTTTACCCGTCCTGTCCTGAGGATGACTTGATAACAAC (SEQ ID NO:84)	HindIII

		NO:84)	
orf8	F	CGCGGATCCGATGTATTATGCCCTTGCCTCTTTCATAAAAAG (SEQ ID NO:85)	BamHI
	R	CGCGGATCCGATGTATTATGCCCTTGCCTCTTTCATAAAAAG (SEQ ID NO:86)	HindIII
sepZ	F	CGCGGATCCGAAGCAGCAAATTTAAGTCCTTC (SEQ ID NO:87)	BamHI
	R	CCCAAGCTTTTAGGCATATTTTCATCGCTAATGCAC (SEQ ID NO:88)	HindIII
orf12	F	CGCGGATCCAATCTTTTAGTTAAAAGAAACGTTG (SEQ ID NO:89)	BamHI
	R	CCCAAGCTTTCATGATGTCATCCTGCGAACG (SEQ ID NO:90)	HindIII
escN	F	CGCGGATCCATTTTCAGAGCATGATTCTGTATTG (SEQ ID NO:91)	BamHI
	R	CGCGGATCCATTTTCAGAGCATGATTCTGTATTG (SEQ ID NO:92)	PstI
orf15	F	CGCGGATCCTTGGACAGAATTTTATCTATTCGT (SEQ ID NO:93)	BamHI
	R	CCCAAGCTTCTAGTCAAAGTAATGTTCTTTATGGC (SEQ ID NO:94)	HindIII
orf16	F	CGCGGATCCGCTTCTTTATGGAAGAGATTGTTTTACTCCTCGGG (SEQ ID NO:95)	BamHI
	R	CCCAAGCTTTTAATTTTCATATTCAATTGTGAACTCAATGGC (SEQ ID NO:96)	HindIII
sepQ	F	CGCGGATCCAAGCCATTGAGTTCACAATTG (SEQ ID NO:97)	BamHI
	R	CCCAAGCTTTTAATCACATACTATGCTAACAG (SEQ ID NO:98)	HindIII
espH	F	CGCGGATCCTCGTTATCAGGAGCGGTATTCAAG (SEQ ID NO:99)	BamHI
	R	CCCAAGCTTTCATAATACGCTATAAGAGGAAGC (SEQ ID NO:100)	HindIII
cesF	F	CGCGGATCCAATGAGAAATTCGCACAGACCTTG (SEQ ID NO:101)	BamHI
	R	CCCAAGCTTTCAAGGTAAAAAATCTGTAGGTCTGG (SEQ ID NO:102)	HindIII
map	F	CGGGGTACCTTTAGTCCAATGACAATGGCAGGC (SEQ ID NO:103)	KpnI
	R	CCCAAGCTTCTACAATCGGGTATCCTGTACATG (SEQ ID NO:104)	HindIII
tir	F	CGGGGTACCCCTATTGGTAATCTTGGTCATAATC (SEQ ID NO:105)	KpnI
	R	CCCAAGCTTTTAGACGAAACGATGGGATCCC (SEQ ID NO:106)	HindIII
cesT	F	CGCGGATCCTCATCAAGATCTGAACTTTTATTAG (SEQ ID NO:107)	BamHI
	R	CCCAAGCTTTTATCTTCCGGCGTAATAATG (SEQ ID NO:108)	HindIII
escD	F	CGCGGATCCTTATCCTCATATAAAATAAAAC (SEQ ID NO:109)	BamHI
	R	CGCGGATCCTTATCCTCATATAAAATAAAAC (SEQ ID NO:110)	HindIII
sepL	F	CGCGGATCCGCTAATGGTATTGAATTTAATC (SEQ ID NO:111)	BamHI
	R	AAACTGCAGTCAAATAATTTCTCCTTATAGTCG (SEQ ID NO:112)	PstI
espA	F	CGCGGATCCGATACATCAAATGCAACATCCGTTG (SEQ ID NO:113)	BamHI
	R	AAACTGCAGTTATTTACCAAGGGATATTGCTG (SEQ ID NO:114)	PstI
espD	F	CGCGGATCCCTTAACGTAAATAACGATACCCTG (SEQ ID NO:115)	BamHI
	R	CGGGGTACCTTAAATTCGGCCACTAACAATACG (SEQ ID NO:116)	KpnI
espB	F	CGCGGATCCAATACTATTGATAATACTCAAGTAACGATGG (SEQ ID NO:117)	BamHI
	R	AAACTGCAGTTACCCAGCTAAGCGACCCGATTGCCCC (SEQ ID NO:118)	PstI
cesD2	F	CGCGGATCCGTCGATACGTTTAATGATGAAGTG (SEQ ID NO:119)	BamHI
	R	AAACTGCAGTTAACTATTTACGTTTATTACGAACC (SEQ ID NO:120)	PstI
escF	F	CGCGGATCCAATTTATCTGAAATTACTCAAC (SEQ ID NO:121)	BamHI
	R	CCCAAGCTTTTAAAACTACGGTTAGAAATGG (SEQ ID NO:122)	HindIII
orf29	F	CGCGGATCCGTTAATGATATTTCTGCTAATAAGATACTGG (SEQ ID NO:123)	BamHI
	R	AAACTGCAGTTAAAATCCTCGTACCCAGCCACTACC (SEQ ID NO:124)	PstI

espF	F	CGCGGATCCCTTAATGGAATTAGTAACGCTGC (SEQ ID NO:125)	BamHI
	R	CCCAAGCTTTTACCCTTTCTTCGATTGCTCATAGG (SEQ ID NO:126)	HindIII
orf1 *	F	CGCGGATCCCCTCACCTCAAGAACTCACTTTC (SEQ ID NO:127)	BamHI
	R	ACGCGTCGACTTACTTATTAGGGACAAATTTC (SEQ ID NO:128)	Sall
espG	F	CGCGGATCCATACTTGTTGCCAAATTGTTC (SEQ ID NO:129)	BamHI
	R	AAACTGCAGTTAAGTGTTTTGTAAGTACGTTTCAGATGCGG (SEQ ID NO:130)	HindIII
non-LEE			
nleA	F	GGAAGATCTAACATTCAACCGACCATACAATC (SEQ ID NO:131)	BglII
	R	TCCCCCGGGTTAGACTCTTGTTTCTTGG (SEQ ID NO:132)	XmaI
nleB	F	CGCGGATCCTTATCTTCATTAAATGTCCTTCAATCCAGC (SEQ ID NO:133)	BamHI
	R	CCCAAGCTTTTACCATGAAGTGCAGGTATACATACTG (SEQ ID NO:134)	HindIII
nleB-1	F	CGCGGATCCCTTTCACCGATAAGGACAACCTTC (SEQ ID NO:135)	BamHI
	R	CGGGGTACCTTACCATGAAGTGCATGTATACTG (SEQ ID NO:136)	KpnI
nleC	F	CGCGGATCCAAAATTCCCTCATTACAGTCCAAC (SEQ ID NO:137)	BamHI
	R	CCCAAGCTTTCATTGCTGATTGTGTTTGTCCAC (SEQ ID NO:138)	HindIII
nleD	F	CGCGGATCCCGCCCTACGTCCCTCAACTTGGTATTAC (SEQ ID NO:139)	BamHI
	R	CCCAAGCTTCTAAAGCAATGGATGCAGTCTTACCTG (SEQ ID NO:140)	HindIII
nleE	F	CGCGGATCCATTAATCCTGTTACTAATACTCAGGGCGTGTCCCC TATAAATACTAAATATGCTGAACATG (SEQ ID NO:141)	BamHI
	R	CCCAAGCTTCTACTCAATTTTAGAAAGTTTATTATTTATGTATTT CATATAACTGTCTATTTCCCCAGGC (SEQ ID NO:142)	HindIII
nleF	F	CGCGGATCCTTACCAACAAGTGGTTCTTCAGC (SEQ ID NO:143)	BamHI
	R	CCCAAGCTTTCATCCACATTGTAAAGATCCTTTG (SEQ ID NO:144)	HindIII
nleG	F	CGCGGATCCCCTGTCATATTAACCTTTTCGAGTG (SEQ ID NO:145)	BamHI
	R	CCCAAGCTTTCAAATTCTAGTGCATATATTTTGTGTGGC (SEQ ID NO:146)	HindIII
nleH1-2	F	CGCGGATCCTTATCGCCCTCTTCTATAAATTTGGGATGTTTCATGG (SEQ ID NO:147)	BamHI
	R	CCCAAGCTTTTATATCTTACTTAATACTACACTAATAAGATCCAGC (SEQ ID NO:148)	HindIII
nleI	F	CGCGGATCCCAGGTTCTTCGTGCTCAAATGG (SEQ ID NO:149)	BamHI
	R	CCCAAGCTTTCATAAATACATTGTTCTTGAC (SEQ ID NO:150)	HindIII
nleG2-1	F	CGCGGATCCAATGTCCTTCGAGCTCAAGTAGCATCTAG (SEQ ID NO:151)	BamHI
	R	CCCAAGCTTTTAACTATCTTTTATAATGAAGTTTCCC (SEQ ID NO:152)	HindIII
nleG2-2	F	CGCGGATCCCCATTAACCTCAGATATTAGATCAC (SEQ ID NO:153)	BamHI
	R	CCCAAGCTTTCAATTACCCTTTATAACGAAGTTTCC (SEQ ID NO:154)	HindIII
nleG3	F	CGCGGATCCGTAATGCCTGGATTAGTATC (SEQ ID NO:155)	BamHI
	R	CCCAAGCTTTTAATGCAATTGAAATAAATAAG (SEQ ID NO:156)	HindIII
nleG5-1	F	CGCGGATCCCCTGTAGATTTAACGCCTTATATTTTACCTGGG (SEQ ID NO:157)	BamHI
	R	CCCAAGCTTTTAATTTTAAACGAAGTTACCTCTGTCAGGG (SEQ ID NO:158)	HindIII
nleG6-1	F	CGCGGATCCCCTGTTACCACCTTAAGTATCCC (SEQ ID NO:159)	BamHI
	R	CGGGGTACCTCACTTACAACAAAAGCTTCTC (SEQ ID NO:160)	KpnI

nleG8-2	F	CGCGGATCCCCAGTCATATTAAATTTTTCTAATGGAAGTG (SEQ ID NO:161)	BamHI
	R	CCCAAGCTTTTAAATACTGTTTTGTTGAAGTGGGTATATG (SEQ ID NO:162)	HindIII
nleG9	F	CGCGGATCCGACGCTTTTATTGTAGATCCTGTTC (SEQ ID NO:163)	BamHI
	R	CCCAAGCTTCTACACTGAATAACAATCACTCC (SEQ ID NO:164)	HindIII
espK	F	CGCGGATCCATGCTTCCTACATCGCAATTACGAC (SEQ ID NO:165)	BamHI
	R	CCCAAGCTTTTAAGAATATTTATATGTGGAACCAGAG (SEQ ID NO:166)	HindIII
espL2	F	CGGATCCCCAATAATAACAAATCGGCATCAAATTATG (SEQ ID NO:167)	BamHI
	R	CCCAAGCTTTCAATTGGAATAATAATTATATACATCGAGG (SEQ ID NO:168)	HindIII
espM2	F	CGCGGATCCCCGATGAATACTACAGGTATGTC (SEQ ID NO:169)	BamHI
	R	CCCAAGCTTTCATCCCTGTATAGCACGCATC (SEQ ID NO:170)	HindIII
espR1	F	CGCGGATCCAAATTCCCTTCAATATTTAACAAAATAAAACC (SEQ ID NO:171)	BamHI
	R	CGGGGTACCTTAGTGATAAAAAGGCCATGAGCTGGAGG (SEQ ID NO:172)	KpnI
tccp	F	CGCGGATCCATTAACAATGTTTCTTCACTTTTICC (SEQ ID NO:173)	BamHI
	R	CCCAAGCTTTCACGAGCGCTTAGATGTATTAATG (SEQ ID NO:174)	HindIII
espV	F	CGCGGATCCAGCGGAACCTCAGGTTCTCG (SEQ ID NO:175)	BamHI
	R	CCCAAGCTTTCACAAAAAAGATTGGGGAGG (SEQ ID NO:176)	HindIII
espW	F	CGCGGATCCCCCAAATATCATCAGTTGTATCATC (SEQ ID NO:177)	BamHI
	R	CCCAAGCTTTTAATTTCTAACCAAGGGGTCCCATG (SEQ ID NO:178)	HindIII
espX2	F	CGCGGATCCGATTGTTCAAAATGCAATGGTTATG (SEQ ID NO:179)	BamHI
	R	CCCAAGCTTTTACAGCCATGCGTCTGGCGTCCAC (SEQ ID NO:180)	HindIII
espX7	F	CGCGGATCCAAACATATAGAAGGTTCTTTCTG (SEQ ID NO:181)	BamHI
	R	CGGGGTACCTCAACGCCACGCAACAGGATAATAC (SEQ ID NO:182)	KpnI
espY1	F	CGCGGATCCAAAGTATCAGTTCCAGGCATGC (SEQ ID NO:183)	BamHI
	R	CCCAAGCTTTCATTCAATAATTGCGTTGTCAG (SEQ ID NO:184)	HindIII
espY2	F	CGCGGATCCAAAGTAAGAAACCCAGAACAGATTAG (SEQ ID NO:185)	BamHI
	R	CCCAAGCTTTCAGTCATACCAACGGCTATTGTTCG (SEQ ID NO:186)	HindIII
espY3	F	CGCGGATCCATGAAAACCATCACCAACAACCG (SEQ ID NO:187)	BamHI
	R	CCCAAGCTTTCAGTCGACGAACCTCATAATAATTGCTC (SEQ ID NO:188)	HindIII

Table 4. Oligonucleotide primers used for the amplification of LEE and non-LEE genes.

Nucleotide sequence is from 5' to 3'. Restriction sites incorporated into the primers are listed. *=GST fused genes.

5

Example 5

Western Blot and ELISAs using Anti-TTSP STEC O157:H7 and Non-O157:H7 Sera

The purified proteins from Example 4 were then tested in Western blots using sera raised against TTSP from STEC O157:H7 and non-O157 serotypes. Western blots were performed on both the LEE Pathogenicity Island proteins and the non-LEE purified proteins using rabbit anti-TTSPs STEC O157:H7, bovine anti-TTSPa STEC O157:H7 and

10

anti-His-tag monoclonal antibodies. Western blots were also performed using sera against TTSPs from STEC O26, O111 and O103. All proteins were run on 12% SDS-PAGE gels.

A total of 20 proteins reacted with serum from at least one serotype. A summary of the reactive proteins are found on Table 5A.

A

Proteins	O157:H7	O26:H11	O103:H2	O111:NM
EscC				
SepD				
Tir				
EspA				
EspD				
EspB				
EspF				
Rorf1				
EspG				
NleA				
NleE				
NleF				
EspR1				
NleH				
NleI				
NleG2-1				
NleG2-2				
EspM2				
Tccp				
EspY1				

B

Proteins	Vaccinated with STEC O157:H7 TTSPs	Experimentally infected with STEC O157:H7
Tir		
EspA		
EspD		
EspB		
EspG		
EspM2		
NleA		
TccP		

Table 5. Summary of reactive recombinant STEC O157 TTSPs against rabbit O26-, O103-, O111- and O157-specific sera, and sera from O157-experimentally infected and O157-vaccinated cattle. A) LEE and non-LEE proteins which reacted against O26-, O103-, O111- and O157-specific sera. O157:H7 = rabbit anti-O157 TTSPs polyclonal antibodies;

(Pre) preimmune sera; O26:H11 = rabbit anti-O26 TTSPs polyclonal antibodies; O103:H2 = rabbit anti-O103 TTSPs polyclonal antibodies; O111:NM = rabbit anti-O111 TTSPs polyclonal antibodies. B) LEE and non-LEE proteins which reacted against sera from O157-experimentally infected and O157-vaccinated cattle. Grey boxes represent positive reactivity.

Recombinant purified STEC O157:H7 proteins were also tested in ELISAs using sera raised against TTSP from STEC O157:H7 and non-O157 serotypes to further confirm results from Western blots. All samples were done in triplicates. The majority of proteins produced identical results to Western blots (positive based on a 2-log difference in titer compared to preimmune) (Table 6). However a number of proteins did not produce matching results or only demonstrated a 1-log difference compared to the preimmune. Proteins Map and NleG6-1 were used as negative controls as these proteins gave negative results on Western blots. These mixed results could be related to the level of denaturation which the proteins go through in Western blots compared to ELISAs.

	157	pre	26	103	111
NleE	6398±131	1151±66	1242±295	2342±494	5648±225
EspD	410558±103216	227±8	5742±120	384613±152955	264134±59212
EspRI	153555±38091	2907±978	6552±303	3595±1619	6744±923
EspY1	1834±86	1926±58	7368±195	6560±3340	6493±334
Tir	569786±11321	425±24	516982±15432	109109±11176	496833±37645
EspF	960±79	335±16	6985±130	30124±8674	84486±14868
NleI	5626±199	412±31	2266±965	23108±6365	5224±230
EscC	6721±270	1539±75	22634±1565	7120±438	17003±1047
NleH	24066±1788	4185±382	5930±191	18694±1033	2597±917
TccP	1447±81	368±14	132429±44422	27261±1093	6875±67
EspM2	6522±707	921±725	4723±1637	6785±122	6064±950
EspA	637500±162376	234±29	297646±53126	299648±133401	395028±14921
EspB	511393±139707	179±27	99719±734	474865±3983	497104±29944
EspG	386863±61345	397±4	5643±352	1123±69	422629±47581
NleA	460507±14720	128±4	6389±1094	55801±43319	20062±2411
NleF	1362±59	314±33	392±23	512229±51334	4155±815
NleG2.1	4566±518	388±9	2587±1555	121235±31162	6563±591
nleG2.2	6719±527	953±695	2573±1422	84860±12521	7027±6
SepD	6453±362	265±28	760±469	197773±47988	1381±49
NleG6-1	4446±137	674±484	1357±189	1476±125	1409±79
Map	4617±161	385±15	470±14	1269±91	1577±105

5 **Table 6. Titre results from ELISAs completed using anti-TTSP STEC O157:H7 and non-O157 sera against recombinant purified STEC O157:H7 proteins.** Data shown as mean ± standard deviation. (157) Rabbit anti-O157 TTSPs polyclonal antibodies; (Pre) preimmune sera; (26) Rabbit anti-O26 TTSPs polyclonal antibodies; (103) Rabbit anti-O103 TTSPs polyclonal antibodies; (111) Rabbit anti-O111 TTSPs polyclonal antibodies.

Example 6

Western Blot and ELISAs using Sera from Experimentally Infected Cattle with STEC

O157:H7

Sera from experimentally infected cattle were also tested against the recombinant
5 purified STEC O157:H7 proteins from Example 4. A total of six proteins reacted with
the experimentally infected sera consisting of Tir, EspA, EspD, EspB, EspM2 and TccP
(Table 5B). The recombinant purified STEC O157:H7 proteins were also tested in
ELISAs using sera from experimentally infected cattle. Single well dilutions of sera were
used for each protein. Preimmune cattle sera was used to calculate background values
10 against each protein. The ELISA OD value was measured by subtracting the preimmune
value from the infected cattle value. Duplicate values were averaged and three standard
deviations were calculated before subtraction.

Of all 66 proteins tested, five proteins gave positive results. See, Figure 13.
Negative proteins not shown in Figure 13 include Ler, Orf2, CesA/B, Orf4, Orf5, EscS,
15 EscT, Rorf13, GrlR, GrlA, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16,
SepQ, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, EspG, NleB,
NleB2-1, NleC, NleE, NleF, NleG, NleH1-2, NleI, NleG2-1, NleG2-2, NleG3, NleG5-1,
NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspM2, EspR1, TccP, EspV, EspW, EspX2,
EspX7, EspY1, EspY2 and EspY3.

20 Four of five positive proteins for ELISAs were also positive in Western blots (Tir,
EspB, EspD and EspA).

Example 7

ELISA Results using Sera from Human HUS Patients

25 A. Sixteen serum samples from positive and negative human patients collected
from the Walkerton outbreak in 2000. Samples were collected two years post-outbreak.
Samples were tested against an immunogenic antigen (Tir) which correlates with infection
by STEC O157:H7 (Figure 14). A set of negative samples were also collected and used
as an extra set of negative samples. Overall, no significant difference was observed with
30 the three sets of serum, meaning that at time of collection antibodies against such antigens

were no longer present.

B. In a second experiment, serum from six additional patients who developed HUS from STEC O157:H7 infection was tested against the 66 recombinant purified *E. coli* O157:H7 proteins. A total of 12 proteins out of 66 tested reacted against the human sera. Single well dilutions of human sera at 1:500 were used for each protein. Naive human sera was calculated to measure the background of each protein. The ELISA OD value was measured by subtracting the naive value from the HUS positive human sera. Duplicate values were averaged and three standard deviations calculated before subtraction.

In general four proteins reacted consistently with the majority of the sera tested (Tir, EspD, EspA and NleA). See, Figure 15. Interestingly, these are the same proteins which reacted against the serum from experimentally infected cattle in Example 6. Negative proteins not shown in Figure 15 include Ler, Orf2, CesA/B, Orf4, Orf5, EscT, Rorf13, GrlR, GrlA, CesD, EscC, SepD, EscJ, Orf8, SepZ, Orf12, EscN, Orf16, EspH, CesF, Map, CesT, EscD, SepL, CesD2, EscF, Orf29, EspF, NleB, NleB2-1, NleC, NleE, NleG, NleH1-2, NleI, NleG2-2, NleG3, NleG5-1, NleG6-1, NleG8-2, NleG9, EspK, EspL2, EspR1, TccP, EspV, EspW, EspX2, EspX7, EspY1, EspY2 and ESpy3.

Example 8

Vaccination of Mice using Recombinant STEC Proteins

Three groups of 10 mice (see below) were vaccinated as follows.

Group 1 -- placebo (0.1 M phosphate buffered saline (PBS))

Group 2 -- O157:H7 TTSPs (TTSPs secreted into M9 media that are a cocktail of mostly unidentified proteins) in 30% EMULSIGEN D (MVP Laboratories, Ralston, NE);

Group 3 -- Recombinant O157:H7 EspG, NleH2-1, NleA, EspRI, EspF, EspB, EspD, EspA and the chimeric Tir described above plus 30% EMULSIGEN D.

Mice were initially vaccinated subcutaneously with 0.5 µg of antigen and blood samples collected. 21 days later, mice were again vaccinated as above and blood samples collected. 19 days later, mice were treated with water containing 5g/L streptomycin for

24 hours to remove normal intestinal flora. Mice were then deprived of food and water for 18 hours, Blood samples were again collected and mice were challenged with a 100 μ l oral dose of 10^9 CFU/ml of nal^r *E.coli* O157 strain in 20% sucrose. Beginning two days later, fecal samples were collected every two days for two weeks and fecal shedding of
5 STEC was examined.

In particular, one pellet of a mouse fecal sample (approximately 0.1 g) was combined with 1 ml Luria broth and incubated at room temperature for 2-4 hours to allow the pellet to soften. The sample was vortexed to disperse the pellet and the sample diluted in PBS and 25 μ l dots were plated in triplicate on CT-SMAC agar plates (Mackonkey agar
10 + Cefiximine 0.05 mg/L + Tellurite 2.5 mg/L + nalidixic acid 15 mg/L). Plates were incubated overnight at 37°C, colonies were counted and the presence of *E. coli* O157 was confirmed by agglutination tests.

Data was summed over time. The sums were not normally distributed so they were log-transformed and one-way ANOVA followed by Tukey's comparison and means
15 test. Results are shown in Figure 26. Medians of raw data were used as data points. There were significant differences among the groups ($P < 0.0001$). The earlier samples taken from both of Groups 2 and 3 had significantly less fecal shedding than Group 1.

Thus, compositions and methods for treating and preventing enterohemorrhagic *E. coli* colonization of mammals have been disclosed. Although preferred embodiments of
20 the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

CLAIMS

1. A multiple epitope fusion protein comprising epitopes from STEC O157:H7 Tir, STEC O26:H11 Tir, STEC O103:H2 Tir and STEC O111:NM Tir, wherein the protein comprises a full-length STEC O157:H7 Tir sequence corresponding to amino acids 1-558 of SEQ ID NO:52; a STEC O111:NM Tir epitope comprising a sequence of amino acids corresponding to amino acids 565-644 of SEQ ID NO:52; a STEC O26:H11 Tir epitope comprising a sequence of amino acids corresponding to amino acids 651-705 of SEQ ID NO:52; and a STEC O103:H2 Tir epitope comprising a sequence of amino acids corresponding to amino acids 712-741 of SEQ ID NO:52.
2. The multiple epitope fusion protein of claim 1, wherein the protein comprises the amino acid sequence of SEQ ID NO:52.
3. The multiple epitope fusion protein of claim 1 or 2, linked to a carrier molecule.
4. The multiple epitope fusion protein of claim 3, wherein the carrier molecule is an RTX toxin.
5. The multiple epitope fusion protein of claim 4, wherein the RTX toxin is a leukotoxin polypeptide.
6. The multiple epitope fusion protein of claim 5, wherein the leukotoxin polypeptide is LKT 352.
7. The multiple epitope fusion protein of claim 6, wherein the protein comprises the amino acid sequence of SEQ ID NO:54.
8. A composition comprising the multiple epitope fusion protein of any one of claims 1-7 and a pharmaceutically acceptable vehicle.
9. A method of producing a composition comprising combining the multiple epitope fusion protein of any one of claims 1-7 with a pharmaceutically acceptable vehicle.
10. A polynucleotide comprising a coding sequence encoding the multiple epitope fusion

protein of any one of claims 1-7.

11. A recombinant vector comprising:

- (a) a polynucleotide according to claim 10; and
- (b) control elements that are operably linked to said polynucleotide whereby said coding sequence can be transcribed and translated in a host cell.

12. A host cell transformed with the recombinant vector of claim 11.

13. A method of producing a multiple epitope fusion protein comprising:

- (a) providing a population of host cells according to claim 12; and
- (b) culturing said population of cells under conditions whereby the protein encoded by the coding sequence present in said recombinant vector is expressed.

14. A method of detecting STEC antibodies in a biological sample comprising:

- (a) providing a biological sample;
- (b) reacting said biological sample with a multiple epitope fusion protein according to any one of claims 1-7 under conditions which allow STEC antibodies, when present in the biological sample, to bind to said multiple epitope fusion protein to form an antibody/antigen complex; and
- (c) detecting the presence or absence of said complex, thereby detecting the presence or absence of STEC antibodies in said sample.

15. An immunodiagnostic test kit for detecting STEC infection, said test kit comprising a multiple epitope fusion protein according to any one of claims 1-7, and instructions for conducting the immunodiagnostic test.

16. The composition of claim 8, further comprising an immunological adjuvant.

17. A composition comprising (a) at least two purified immunogenic Shiga toxin-producing *Escherichia coli* (STEC) proteins selected from the group consisting of Tir, EspA, EspB, EspD, EspR1, N1eA, Tccp, EspG, EspF, N1eE, N1eA, N1eH and N1eH2-1, wherein the STEC proteins are selected from a full-length STEC protein, an immunogenic fragment or variant thereof, wherein at least one of the STEC proteins generates antibodies that react with STEC

0157 and at least one other STEC serotype; and (b) the multiple epitope fusion protein of any one of claims 1-7.

18. The composition of claim 17, further comprising an immunological adjuvant.

19. The multiple epitope fusion protein of any one of claims 1-7, for use in eliciting an immunological response in a mammal against a STEC antigen.

20. The composition of 8, 16, 17, or 18, for use in eliciting an immunological response in a mammal against a STEC antigen.

21. Use of the composition of claim 8, 16, 17, or 18, for reducing colonization of STEC in a ruminant.

22. Use of the multiple epitope fusion protein of any one of claims 1-7 for reducing colonization of STEC in a ruminant.

23. Use of the composition of claim 8, 16, 17, or 18, in the manufacture of a medicament for reducing colonization of STEC in a ruminant.

24. Use of the multiple epitope fusion protein of any one of claims 1-7 in the manufacture of a medicament for reducing colonization of STEC in a ruminant.

25. Use of the composition of claim 8, 16, 17, or 18, for reducing shedding of STEC from a ruminant.

26. Use of the multiple epitope fusion protein of any one of claims 1-7, for reducing shedding of STEC from a ruminant.

27. Use of the composition of claim 8, 16, 17, or 18, in the manufacture of a medicament for reducing shedding of STEC from a ruminant.

28. Use of the multiple epitope fusion protein of any one of claims 1-7 in the manufacture of a medicament for reducing shedding of STEC from a ruminant.

29. Use of the composition of claim 8, 16, 17, or 18, for eliciting an immunological response in a mammal against a STEC antigen.

30. Use of the multiple epitope fusion protein of any one of claims 1-7, for eliciting an immunological response in a mammal against a STEC antigen

31. Use of the composition of claim 8, 16, 17, or 18, for the preparation of a medicament for eliciting an immunological response in a mammal against a STEC antigen.

32. Use of the multiple epitope fusion protein of any one of claims 1-7, for the preparation of a medicament for eliciting an immunological response in a mammal against a STEC antigen.

33. The use of any one of claims 29-32, wherein the mammal is a ruminant.

34. The use of claim 33, wherein the ruminant is a bovine subject.

35. Use of the multiple epitope fusion protein according to any one of claims 1-7 for detecting STEC antibodies in a biological sample in order to determine the presence of infection.

36. A multiple epitope fusion protein comprising epitopes from more than one Shiga toxin-producing *Escherichia coli* (STEC) serotype, wherein the protein comprises an amino acid sequence with at least 95% identity to the contiguous amino acid sequence set forth in SEQ ID NO: 52.

37. The multiple epitope fusion protein of claim 36, wherein the protein comprises the amino acid sequence set forth in SEQ ID NO: 52.

38. The multiple epitope fusion protein of claim 36 or 37, linked to a carrier molecule

39. The multiple epitope fusion protein of claim 38, wherein the carrier molecule is an RTX toxin.

40. The multiple epitope fusion protein of claim 39, wherein the RTX toxin is a leukotoxin polypeptide.

41. The multiple epitope fusion protein of claim 40, wherein the leukotoxin polypeptide is LKT 352.

42. The multiple epitope fusion protein of claim 41, wherein the protein comprises an amino acid sequence with at least 95% identity to the contiguous amino acid sequence set forth in SEQ ID NO: 54.

43. The multiple epitope fusion protein of claim 42, wherein the protein comprises the amino acid sequence set forth in SEQ ID NO: 54.

44. A composition comprising the multiple epitope fusion protein of any one of claims 36-43, and a pharmaceutically acceptable vehicle.

45. A method of producing a composition comprising combining the multiple epitope fusion protein of any one of claims 36-43 with a pharmaceutically acceptable vehicle.

46. A method of detecting antibodies directed against a STEC serotype in a biological sample comprising:

- (a) providing a biological sample;
- (b) reacting said biological sample with the multiple epitope fusion protein according to any one of claims 36-43 under conditions which allow antibodies against the STEC serotype, when present in the biological sample, to bind to said multiple epitope fusion protein to form an antibody/antigen complex; and
- (c) detecting the presence or absence of said complex, thereby detecting the presence or absence of antibodies against the STEC serotype in said sample.

47. An immunodiagnostic test kit for detecting STEC infection, said test kit comprising a multiple epitope fusion protein according to any one of claims 36-43, and instructions for conducting the immunodiagnostic test.

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1  ttagacgaaa cgatgggata cgggcgctgg tgggttattc gaagtattca cagcgctatt
61 actccccccc gttaatcctc ccatgtcatg gcgtaatcca ccacttagcg ccagacgcgc
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181 atccccggga ggatggtgaa tggtgctata tacaacagaa tctgtattcc ccatattctg
241 aacagacgta ttagaattag aagtcggcac ctgcgaatca tgcagcgatg ttttaacatc
301 agcatacgga ttctgcacgg tccctatgct ggaagtgtca aagaaagtcg acgaggtgct
361 agccatcgag ctacgtctgc tctccatggg atcttctgac ccaggggtat ctacattgcc
421 ctgtgcaggt gtattatttg caggcttatt ctctaccgta cgtgcgcttg tagttgtagt
481 tgtagtagtt gttgttggtg tttgttctac cggctgattt tttcgatgaa gcgcagcggg
541 gacggcaaca ccaattcccc caccaagaat caatgcgcca ctaagaccgt agccagcccc
601 cgatgaaact ttcagctcct cctggcggtt agcttggtgt tcatcatatt ttttttgcgc
661 ctgagcatta ttttcaatgg cttgctggtt ggcctcttcg cctgctgctt tagcctgctc
721 ttctatatte gcaacaacat catctttcaa tacccttgac ggaatcgcat ttccgagctc
781 atcgatatta actttttgat tatctggggt ctggaacgct tctttcgtta actgatctct
841 tgctgcagtt tcagttgcac ttgcagctgc atcagggctg gtcgtggttg ggctatccgg
901 ctccggcgct aatgcaagcg cctgtacaat acccgctgcc gccaacccca tcagacctgt
961 agcaacagtc cccaacgcca accaaagttt aggatctgaa cgaaggctgg aagttgaggt
1021 tgaggtctga gtttctgtgg tgttttccgc accgctattt gactccctca actccccaac
1081 gccttttgac tccccagcac ctttggactc cccggtecc tttgggtcta acagctccag
1141 tatcctttgg cgggcttccg tgatatctga agcaacgggt accatagcat gccagcacc
1201 accacggcct ccagtaaata caaatgtgc tttaccttca ggatcaatgg actgcaagcg
1261 agcgtactct tgatcactta aaacaacaga ggtctcaaca ccattcctct gaccgacagc
1321 aatatgttta ccatcttcct gagtttcaac tcgaaatacc gaagagccaa tctgcctgtt
1381 aagagtatcg agcggaccat gatcatgaag aacttcaaat ccatcattca gtgttatctc
1441 agacgccgcc aggcgcacgt gatttacagg aagtccagga acatcactgg cacgattgtc
1501 gccagaatca gccatagaat tccttacagg cgtaaatagc gcacgagatc ccaacggccc
1561 cgtagagtta atgagctgac cacgcccccc tgcaccgtcg gtttgtgaag gtaatggagg
1621 tgcaggagga attgaattat tcacattggg attatgacca agattaccaa taggcat
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A

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1  MPIGNLGHNPVNNNSIPPAPPLPSQTDGAGGRGQLINSTGPLGSRALFTP
51  VRNSMADSGDNRASDVPLPVNPMRLAASEITLNDGFEVLHDHGPLDTLN
101 RQIGSSVFRVETQEDGKHIAVGQRNGVETSVVLSDDQYARLQSIDPEGKD
151 KFVFTGGRGGAGHAMVTVASDITEARQRILELLEPKGTGESKGAGESKGV
201 GELRESNSGAENTTETQTSTSTSSLRSDPKLWLALGTVATGLIGLAATGI
251 VQALALTPEPDSPTTTDPDAAASATETATRDQLTKEAFQNPQNQKVNIDE
301 LGNAIPSGVLKDDVVANIEEQAKAAGEEAKQQAIENNAQAQKKYDEQQAK
351 RQEELKVSSGAGYGLSGALILGGGIGVAVTAALHRKNQPVEQTTTTTTTT
401 TTTSARTVENKPANNTPAQGNVDTPGSEDTEMESRRSSMASTSSTFFDTSS
451 IGTVQNPYADVKTSLHDSQVPTSNSNTSVQNMGNTDSVVYSTIQHPPRDT
501 TDNGARLLGNPSAGIQSTYARLALSGGLRHDMGGLTGGSNSAVNTSNNPP
551 APGSHRFV
```

B**FIGURE 1**

```

1  atgcctattg gtaatcttgg ccacaatccc aatgtgagag ctttaattcc acctgcaccg
61  ccattacctt cacaaaccga cggcgcagga ggtgcccgtg atcagctcat taactcaaat
121 ggcccgatgg ggtctcgttt gctatttacg cctataagga attctgttgc tgatgctgct
181 gattctcgtg ccagtgatat tcccggactt cctacaaatc cactgcgctt tgctgcgtcc
241 gaggtatctt tgcattggtgc gcttgaagtt cttcatgata aaggggggct tgatactctt
301 aactctgcta ttggatcttc gttattccgt gttgaaactc gggatgatgg cagccatggt
361 gctatcgggc aaaaaaatgg cctcgagacc actgttggtt taagtgagca agagttttct
421 agcttacagt cccttgatcc tgaaggtaaa aacaaatttg tatttactgg aggccgcggt
481 ggcgaggggc atgctatggt cacgggtgct tcagatatcg ccgaagcccg tcagaggata
541 atagataaat tagaaccaaa ggatacaaa gagacgaagg agccagggga tccaaatagt
601 ggcgagggga aaatcattga aattcatacc tcaacctcaa cttctagcct ccgtgcagat
661 cctaaacttt ggttgctcatt ggggactatt gctgcaggtc tgatagggat ggctgcgacg
721 gggattgcac aggctgttgc gttgactcca gagccggatg acccaatcac taccgaccct
781 gatgctgcag caaacacagc tgaagcagcg gcaaaagatc agttaacgaa agaagcattc
841 cagaacccag ataaccagaa agttaatatc gatgagaacg gaaatgcaat tccgtccggg
901 gaactaaaag atgatgttgt tgcgcaaata gcagaacaag ctaaagcggc ggggtgaacg
961 gccagacagg aagctattga aagtaattct caggcgcagc aaaaatatga tgaacagcat
1021 gctaaacgcg aacaggaaat gtctctttca tcggggggtg gctacgggat tagtggtgcg
1081 ctgattcttg gcgggggaat tgggtgccgg gttactgctg ctcttcacgc gaaaaacca
1141 ccggcagAAC aaacaatcac tacacgtacg gtagtcgata atcagcctac gaataacgca
1201 tctgcgcagg gcaataactga cacaagtggg ccagaagagt ccccggcgag cagacgtaat
1261 tcgaatgccA gcctcgcacg gaacgggtct gacacctcca gcacgggcac ggtagagaat
1321 ccgtatgctg acgttggaat gccagaaat gattcactgg ctcgcatttc agaggaacct
1381 atttatgatg aggtcgcctg agatccta attagcgta ttcaacattt ttcaggaac
1441 agcccagtta ccggaagggt agtggaacc ccagggaag gtatccaaag tacttatgcg
1501 cttctggcaa gcagcggcgg attgcgttta ggtatgggag gattaacggg ggggggcgag
1561 agcgcagtaa gtactgcaa tgccgcacca acgccgggac ccgcacgttt cgttttaa

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A

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1      MPIGNLGHNPVRLIPPAPPLPSQTDGAGGARNQLINSNGPMGSRLLEFT
51     PIRNSVADAADSRA SDIPGLPTNPLRFAASEVSLHGALEVLHDKGGLDTL
101    NSAIGSSLFRVETRDDGSHVAIGQKNGLETTVVLSEQEFSSLQSLDPEGK
151    NKFVFTGGRGGAGHAMVTVASDIAEARQRIIDKLEPKDTKETKEPGDPNS
201    GEGKIIIEIHTSTSTSSLRADPKLWLSLGTIAAGLIGMAATGIAQAVALT
251    EPDDPITTPDAAANTAEAAAKDQLTKEAFQNP DNQKVNIDENGNAIPSG
301    ELKDDVVAQIAEQAKAAGEQARQEAI ESNSQAQQKYDEQHAKREQEMSLS
351    SGVGYGISGALILGGGIGAGVTAALHRKNQPAEQ TITTRTVVDNQPTNNA
401    SAQGN TDTSGPEESPASRRNSNASLASNGSDTSSTGTVENPYADVGM PRN
451    DSLARISEEPIYDEVAADPNYSVIQHFSGN SPVTGRLVGTPGQGIQSTYA
501    LLASSGGLRLGMGGLTGGGESAVSTANAAPT PGPARFV

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B**FIGURE 2**

```

1  ttaaacgaaa cgtgcggggtc ccggcggttg tgaggcattg gcagtactta ctgcgctctc
61  gccccccccc gttaatcctc ccatacctaa acgcaatccg ccgctgcttg ccagaagcgc
121 ataagtactt tggatgcctt gccctggggt tccactaac cttccggtaa ctgggctggt
181 ccctgaaaaa tgttgaatga cgctataatt aggatctgca gcgacctcat cataaatagg
241 ttcctctgga atacgagcca gtgaatcatt tctgggcatt ccaacgtcag catacggatt
301 ctctaccgtg cccgtactgg aggtatcaga cccgttcgat gcaaggctgg cattcgaatt
361 gcgcctgctc gccggggact cttctggccc acttgtgtca gtattgccct gtgcagatgc
421 gttattcgta ggctgattat cgactactgt acgtgtagtg attgtttggt ctgccggttg
481 gtttttccga tgaagagcag cagtaacacc ggcaccaatt cccccgcaa gaatcagcgc
541 cccactaata ccgtagccaa ccccgatga aagagacatt tcctgttcgc gtttagcatg
601 ctgctcatca ttttttttct gcgcctgaga attactttca atagcttcct gtctggcctg
661 ttcccccgcc gcttttagctt gttctgctat ttgcgcaaca acatcatcta ttaattcccc
721 tgacggaatt gcatttccgt tctcatcgat attaaacttt tgtttatctg gatcctggaa
781 tgccctctgc gttaaccgat cttttgtcgc agcttcagct gtgcttgctg cagtatcagg
841 atcggtagta gttggatcat ccggtctctg agtcaacgca acagcctgtg caataccgct
901 cgcagccatc cctatcagac ctgcagcaat agtccccaat gacaaccaa gtttaggatc
961 tgcacggagg ctagaagttg aggttgaggt atgaatttca atgatttttc cctcgccact
1021 atttggatcc cctggctcct tcgtctcctt tgtatccttt gggttctaatt tatctattat
1081 cctctgacgg gcttcggcga tatctgaagc aaccgtgacc atagcatgcc ctgcgccacc
1141 gcggcctcca gtaaatacaa atttgttttt accttcagga tcaagggact gtaagctaga
1201 aaactcttga tcacttaaaa caacagtggc ctcgaggcca tttttttgcc cgatagcaac
1261 atggctgcca tcatcccgag tttcaacacg gaataacgaa gatccaatag cagagttaag
1321 agtatcaagc ccccctttat catgaagaac ttcaagcgca ccatgcaaag atacctcgga
1381 cgcagcaaag cgcagtggat ttgtaggaag tccgggaata tcaactggac gagaatcagc
1441 agcatcagca acagaattcc ttataggcgt aaatagcaaa cgagaccca tcgggccatt
1501 tgagttaatg agctgattac gggcacctcc tgcaccgtcg gtttgtgaag gtaatggcgg
1561 tgcaggtgga attaaagctc tcacattggg attgtggcca agattaccaa taggcat

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A

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1  MPIGNLGHNPVRLIPPAPPLPSQTDGAGGARNQLINSNGPMGSRLLEFT
51  PIRNSVADAADSRASDIPGLPTNPLRFAASEVSLHGALEVLHDKGGLDTL
101 NSAIGSSLFRVETRDDGSHVAIGQKNGLETTTVVLSDQEFSSLQSLDPEGK
151 NKFVFTGGRGGAGHAMVTVASDIAEARQRIIDKLEPKDTKETKEPGDPNS
201 GEGKIIIEIHTSTSTSSLRADPKLWLSLGTIAAGLIGMAATGIAQAVALT
251 EPDDPTTTDPDTAASTAEAA TKDRLTQEA FQDPDKQKVNIDENGNAIPSG
301 ELIDDVVAQIAEQAKAAGEQARQEAI ESNSQAQKKYDEQHAKREQEMSLS
351 SGVGYGISGALILGGGIGAGVTAALHRKNQPAEQTITTRTVVDNQPTNNA
401 SAQGNTDTS GP EESPASRRNSNASLASNGSDTSSTGTVENPYADVGM PRN
451 DSLARIPEEPIYDEVAADPNYSVIQH FSGNSPVTGRLVGTPGQGIQSTYA
501 LLASSGGLRLGMGGLTGGGESAVSTANASPTPGPARFV

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B**FIGURE 3**

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1  atgcctattg gtaaccttgg taataatgta aatagcaata atttaattcc gcctgcgccg
61  ccactacctt cacaacacaga cggcgcggtca cggggaggag cgggtcaact aattaactct
121 acaggagcat taggatctcg tttattgttt tctcccctga gaaattctat agctgattct
181 gtcgattcca gagatattcc aggacttcct gtacacccat cgaggcttgc tactgctaca
241 tcagagatat gcttgcttgg aggatttgaa gttctccatg ataagggacc acttgatact
301 ctcaataagc aaattggagc ctctgcattt cgtattgaac agcagtcaga tggttcttat
361 gccgctattg gagaaaaaaa tgggtgtagag gttagcgtta tattaaattc tcaagaattg
421 caaagcttgc aagctatcga tattgaggat aaaggccgat ttgtttttac cgggggacgt
481 ggtggtggtg ggcattccat ggtcactcct gcatcagata tcgcagaagc tcgtgcgaaa
541 atactggcca aattagaccc aaacaatcat gggggaagtc aagccaggaa cgttgatacg
601 cgttctgttg gtgttggaag tgcttcggga atggatgata gcgttggttag cgaaactcgt
661 acttcatcaa cagcttccag cgttcgttca gatcctaaat tctgggtttc tatcggcgca
721 attgctgctg gtttagcggg gctggcggct actggtatta cacaggcgtt ggctttgaca
781 ccggaaccgg atgatcctac aaccaccgat cctgagcagg ctgcaagtgc tgcagaaagt
841 gcgacaagag atcagttaac gcaagaagca ttcaagaatc ctgagaacca gaaagttagc
901 attgatgaga tcggaaattc tattccgtct ggggaattaa aagatgatgt tgttgctaaa
961 atagaagaac aagctaaaga ggcgggtgag gcggccagac agcaggctgt tgaaagcaat
1021 gcacaggcgc agcagcgata tgatactcag tatgccagac gtcaggagga attagagctt
1081 tcatcgggta ttggttacag cctcagcagt gcattgattg ttggtggggg aattggtgct
1141 ggtgtaacga ctgcgcttca tagacgaaat cagccggcag aacaaacaac gacaacaaca
1201 acacatacgg tagtgcagca gcagaccgga gggaaatccc cagcacaagg tggcactgat
1261 gccataagag cggaagacac atctctgaat agacgtgatt cgcagaggag tacggcatcg
1321 acacactggt cagatacttc tagcgcagtg gttaatccat atgctgaagt tggggaggct
1381 cggaatagtt caccggctcg tcaggcagaa gagcatattt acgatgaggt cgctgcagat
1441 cctaattata gcgtcattca aaatttctca gggaataacc aagttaccgg aaggttaatg
1501 ggaactccag ggcaaggtat ccaaagtact tatgcgattc tgacaaacaa cagcgctgga
1561 ttgcgttttag gtatgggtgg attaacgggg agtggcggga gcgcagtaaa tactgcaaat
1621 gccgcaccaa cgccgggacc aggacgtttc gtttaa

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A

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1      MPIGNLGNNVNSNNLI PPAPPLPSQTDGASRGGAGQLINSTGALGSRLLF
51     SPLRNSIADSVDSRDIPGLPVHPSRLATATSEICLLGGFEVLHDKGPLDT
101    LNKQIGASAFRIEQQSDGSYAAIGEKNGVEVSVILNSQELQSLQAIDIED
151    KGRFVFTGGRGGGGHSMVTPASDIAEARAKILAKLDPNNHGGSQARNVDT
201    RSVGVGSA SGMDDSVVSETRTSSTASSVRSDPKFWV SIGAIAAGLAGLAA
251    TGITQALALTPEPDDPTTTDPEQAASAAESATRDQLTQEAFKNPENQKVS
301    IDEIGNSIPSGELKDDVVAKIEEQAKEAGEAARQQAVESNAQAQQRYDTQ
351    YARRQEELELSSGIGYSLSSALIVGGGIGAGVTTALHRRNQPAEQTTTTT
401    THTVVQQQTGGNTPAQGGTDAIRAEDTSLNRRDSQRSTASTHWSDTSSAV
451    VNPYAEVGEARNSSPARQAEHIYDEVAADPNYSVIQNFSGNNQVTGRLM
501    GTPGQGIQSTYAILTNN SAGLRLGMGGLTGSGGSAVNTANAAPTGPGRF
551    V

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B**FIGURE 4**

ATGCCTATTGGTAACCTTGGTCATAATCCCAATGTGAATAATTCAATTCCTCCTGCACCTCCATTACCTTCACAAAC
CGACGGTGCAGGGGGGCGTGGTCAGCTCATTAACCTCTACGGGGCCGTTGGGATCTCGTGCGCTATTTACGCCTGTAA
GGAATTCTATGGCTGATTCTGGCGACAATCGTGCCAGTGATGTTCTGGACTTCCTGTAAATCCGATGCGCCTGGCG
GCGTCTGAGATAACACTGAATGATGGATTTGAAGTTCTTCATGATCATGGTCCGCTCGATACTCTTAACAGGCAGAT
TGGCTCTTCGGTATTTTCGAGTTGAAACTCAGGAAGATGGTAAACATATTGCTGTCCGTCAGAGGAATGGTGTGAGA
CCTCTGTTGTTTTAAGTGATCAAGAGTACGCTCGCTTGCAGTCCATTGATCCTGAAGGTAAAGACAAATTTGTATTT
ACTGGAGGCCGTGGTGGTGCTGGGCATGCTATGGTCACCGTTGCTTCAGATATCACGGAAGCCCGCCAAAGGATACT
GGAGCTGTTAGAGCCCAAAGGGACCGGGGAGTCCAAAGGTGCTGGGGAGTCAAAGGCGTTGGGGAGTTGAGGGAGT
CAAATAGCGGTGCGGAAAACACCACAGAACTCAGACCTCAACCTCAACTTCCAGCCTTCGTTTCAGATCCTAACTT
TGGTTGGCGTTGGGGACTGTTGCTACAGGTCTGATAGGGTTGGCGGCGACGGGTATTGTACAGGCGCTTGCATTGAC
GCCGGAGCCGGATAGCCCAACCACGACCGACCCTGATGCAGCTGCAAGTGCAACTGAAACTGCGACAAGAGATCAGT
TAACGAAAGAAGCGTTCAGAACCCAGATAATCAAAAAGTTAATATCGATGAGCTCGGAAATGCGATTCCGTCAGGG
GTATTGAAAGATGATGTTGTTGCGAATATAGAAGAGCAGGCTAAAGCAGCAGGCGAAGAGGCCAAACAGCAAGCCAT
TGAAAATAATGCTCAGGCGCAAAAAAATATGATGAACAACAAGCTAAACGCCAGGAGGAGCTGAAAGTTTCATCGG
GGGCTGGCTACGGTCTTAGTGGCGCATTGATTCTTGGTGGGGGAATTGGTGTGCGCTCACCGCTGCGCTTCATCGA
AAAAATCAGCCGGTAGAACAAACAACAACACTACTACTACAACTACAAGCGCACGTACGGTAGAGAATAA
GCCTGCAAATAATACACCTGCACAGGGCAATGTAGATACCCCTGGGTCAGAAGATAACCATGGAGAGCAGACGTAGCT
CGATGGCTAGCACCTCGTCGACTTTCTTTGACACTTCCAGCATAGGGACCGTGCAGAATCCGTATGCTGATGTTAAA
ACATCGCTGCATGATTTCGCAGGTGCCGACTTCTAATTCTAATACGTCTGTTTCAGAATATGGGGAATACAGATTCTGT
TGTATATAGCACCATTCAACATCCTCCCCGGGATACTACTGATAACGGCGCACGGTTATTAGGAAATCCAAGTGCGG
GGATTCAAAGCACTTATGCGCGTCTGGCGCTAAGTGGTGGATTACGCCATGACATGGGAGGATTAACGGGGGGGAGT
AATAGCGCTGTGAATACTTCGAATAACCCACCAGCGCCGGGATCCCATCGTTTTCGTCGGTTCTGGCTCCACCGGTGA
AAGTGCGACAAGAGATCAGTTAACGCAAGAAGCATTCAAGAATCCTGAGAACCAGAAAGTTAGCATTGATGAGATCG
GAAATTCTATTCCGTCTGGGGAATTAAAGATGATGTTGTTGCTAAAATAGAAGAACAAGCTAAAGAGGCGGGTGAG
GCGGCCAGACAGCAGGCTGTTGAAAGCAATGCACAGGCGCAGCAGCGATATGATACTCAGTATGCCAGACGTCAGGA
GGAATTATCAGGATCGGGGACTAGTGCACAGGCTGTTGCGTTGACTCCAGAGCCGGATGACCCAATCACTACCGACC
CTGATGCTGCAGCAAACACAGCTGAAGCAGCGGCAAAAGATCAGTTAACGAAAGAAGCATTCCAGAACCCAGATAAC
CAGAAAGTTAATATCGATGAGAACGGAAATGCAATTTTCATCCGGCGGAATGCATGGGGAACAGGCCAGACAGGAAGC
TATTGAAAGTAATTCTCAGGCGCAGAAAAAATATGATGAGCAGCATGCTAAACGCGAACAGGAAATGAAGCTTAATT
AG

A

1 MPIGNLGHNPVNNNSIPPAPPLPSQTDGAGGRGQLINSTGPLGSRALFTPVRNSMADSGD
61 NRASDVPGLPVNPMLRAASEITLNDGFEVLHDHGPLDTLNRQIGSSVFRVETQEDGKHIA
121 VGQRNGVETSVVLSDQEYARLQSIDPEGKDKFVFTGGRGGAGHAMVTVASDITEARQRIL
181 ELLEPKGTGESKGAESKGVGELRESNSGAENTTETQTSTSTSSLRSDPKLWLALGTVAT
241 GLIGLAATGIVQALALTPEPDSPTTTDPDAAASATETATRDQLTKEAFQNPDNQKVNIDE
301 LGNAIPSGVLKDDVVANIEEQAKAAGEEAKQQAIENNAQAQKKYDEQQAQRQEELKVSSG
361 AGYGLSGALILGGGIGVAVTAALHRKNQVEQTTTTTTTTTTTTTSARTVENKPANNTPAQG
421 NVDTPGSEDTEMESRRSSMASTSSTFFDTSSIGTVQNPYADVKTSLHDSQVPTSNSNTSVQ
481 NMGNTDSVVYSTIQHPPRDTTDNGARLLGNPSAGIQSTYARLALSGGLRHDMGGLTGGSN
541 SAVNTSNNPPAPGSHRFVGSSTGESATRDQLTQEAFKNPENQKVSIDEIGNSIPSGELK
601 DDVVAKIEEQAKEAGEAARQQAVESNAQAQQRYDTQYARRQEELSGSGTSAQAVALTPEP
661 DDPITTDPDAAANTAEAAAKDQLTKEAFQNPDNQKVNIDENGNAISSGGMHGEQARQEAI
721 ESNSQAQKKYDEQHAKREQEMKLN

B**FIGURE 5**

ATGGCTACTGTTATAGATCTAAGCTTCCCAAAAACCTGGGGCAAAAAAATTATCCTCTATATTCCCAAAAATTAC
 CAATATGATACTGAACAAGGTAATGGTTTACAGGATTTAGTCAAAGCGGCCGAAGAGTTGGGGATTGAGGTACAA
 AGAGAAGAACGCAATAATATTGCAACAGCTCAAACCAGTTTAGGCACGATTCAAACCGCTATTGGCTTAACTGAG
 CGTGGCATTGTGTTATCCGCTCCACAAATTGATAAATTGCTACAGAAAACCTAAAGCAGGCCAAGCATTAGGTTCT
 GCCGAAAGCATTGTACAAAATGCAAATAAAGCCAAAACCTGTATTATCTGGCATTCAATCTATTTTAGGCTCAGTA
 TTGGCTGGAATGGATTTAGATGAGGCCTTACAGAATAACAGCAACCAACATGCTCTTGCTAAAGCTGGCTTGGAG
 CTAACAAATTCATTAATTGAAAATATTGCTAATTCAGTAAAAACACTTGACGAATTTGGTGAGCAAATTAGTCAA
 TTTGGTTCAAAAACCTACAAAATATCAAAGGCTTAGGGACTTTAGGAGACAAACTCAAAAATATCGGTGGACTTGAT
 AAAGCTGGCCTTGGTTTAGATGTTATCTCAGGGCTATTATCGGGCGCAACAGCTGCACTTGTACTTGCAGATAAA
 AATGCTTCAACAGCTAAAAAAGTGGGTGCGGGTTTTGAATTGGCAAACCAAGTTGTTGGTAATATTACCAAAGCC
 GTTCTTCTTACATTTTAGCCCAACGTGTTGCAGCAGGTTTATCTTCAACTGGGCCTGTGGCTGCTTTAATTGCT
 TCTACTGTTTCTCTTGCGATTAGCCCATTAGCATTTGCCGGTATTGCCGATAAATTTAATCATGCAAAAAGTTTA
 GAGAGTTATGCCGAACGCTTTAAAAAATTAGGCTATGACGGAGATAATTTATTAGCAGAATATCAGCGGGGAACA
 GGGACTATTGATGCATCGGTTACTGCAATTAATACCGCATTTGGCCGCTATTGCTGGTGGTGTGTCTGCTGCTGCA
 GCCGGCTCGGTTATTGCTTCACCGATTGCCTTATTAGTATCTGGGATTACCGGTGTAATTTCTACGATTCTGCAA
 TATTCTAAACAAGCAATGTTTGAGCACGTTGCAAATAAAATTCATAACAAAATTGTAGAATGGGAAAAAATAATC
 ACGGTAAGAACTACTTTGAAAATGGTTACGATGCCCGTTATCTTGCGAATTTACAAGATAATATGAAATTCCTTAC
 TGAACCTTAAACAAAGAGTTACAGGCAGAACGTGTCATCGCTATTACTCAGCAGCAATGGGATAACAACATTGGTG
 ATTTAGCTGGTATTAGCCGTTTAGGTGAAAAAGTCCTTAGTGGTAAAGCCTATGTGGATGCGTTTGAAGAAGGCA
 AACACATTAAAGCCGATAAATTAGTACAGTTGGATTCGGCAAACGGTATTATTGATGTGAGTAATTCGGGTAAAG
 CGAAAACCTCAGCATATCTTATTCAGAACGCCATTATTGACGCCGGGAACAGAGCATCGTGAACGCGTACAAACAG
 GTAAATATGAATATATTACCAAGCTCAATATTAACCGTG TAGATAGCTGGAAAATTACAGATGGTGCAGCAAGTT
 CTACCTTTGATTTAACTAACGTTGTTTCAGCGTATTGGTATTGAATTAGACAATGCTGGAAATGTAACATAAACCA
 AAGAAACAAAAATTATTGCCAACTTGGTGAAGGTGATGACAACGTATTTGTTGGTTCTGGTACGACGGAAATTG
 ATGGCGGTGAAGGTTACGACCGAGTTCCTATAGCCGTGGAAACTATGGTGCTTTAACTATTGATGCAACCAAAG
 AGACCGAGCAAGGTAGTTATACCGTAAATCGTTTCGTAGAAACCGGTAAAGCACTACACGAAGTGACTTCAACCC
 ATACCGCATTAGTGGGCAACCGTGAAGAAAAAATAGAATATCGTCATAGCAATAACCAGCACCATTGCCGGTTATT
 ACACCAAAGATACCTTGAAAGCTGTTGAAGAAATTATCGGTACATCACATAACGATATCTTTAAAGGTAGTAAGT
 TCAATGATGCCTTTAACGGTGGTGTGATGGTGTGATACTATTGACGGTAACGACGGCAATGACCGCTTATTTGGTG
 GTAAAGGCGATGATATTCTCGATGGTGGAAATGGTGTGATGATTTTATCGATGGCGGTAAAGGCAACGACCTATTAC
 ACGGTGGCAAGGGCGATGATATTTTCGTTACCGTAAAGGCGATGGTAATGATATTATTACCGATTCTGACGGCA
 ATGATAAATTATCATTCTCTGATTCGAACTTAAAGATTTAACATTTGAAAAAGTTAAACATAATCTTGTCATCA
 CGAATAGCAAAAAAGAGAAAGTGACCATTCAAAACCTGGTTCCGAGAGGCTGATTTTGCTAAAGAAGTGCCTAATT
 ATAAAGCAACTAAAGATGAGAAAATCGAAGAAATCATCGGTCAAATGGCGAGCGGATCACCTCAAAGCAAGTTGA
 GATCTTATCGCAAAAGGTAACGGCAAAATTACCCAAGATGAGCTATCAAAGGTTGTTGATAACTATGAATTGCTC
 AAACATAGCAAAAATGTGACAAACAGCTTAGATAAGTTAATCTCATCTGTAAAGTGCATTTACCTCGTCTAATGAT
 TCGAGAAATGTATTAGTGGCTCCAACCTCAATGTTGGATCAAAGTTTATCTTCTCTTCAATTTGCTAGGGGATCT
 CCTATTGGTAATCTTGGTCATAATCCCAATGTGAATAATTCAATTCCTCCTGCACCTCCATTACCTTCACAAACC
 GACGGTGCAGGGGGGCGTGGTCAGCTCATTAACCTCTACGGGGCCGTTGGGATCTCGTGCGCTATTTACGCCTGTA
 AGGAATTCTATGGCTGATTCTGGCGACAATCGTGCCAGTGATGTTCCCTGGACTTCCTGTAAATCCGATGCGCCTG
 GCGGCGTCTGAGATAACACTGAATGATGGATTTGAAGTTCTTCATGATCATGGTCCGCTCGATACTCTTAACAGG
 CAGATTGGCTCTTCGGTATTTTCGAGTTGAAACTCAGGAAGATGGTAAACATATTGCTGTGCGGTGAGAGGAATGGT
 GTTGAGACCTCTGTTGTTTTAAGTGATCAAGAGTACGCTCGCTTGCAAGTCCATTGATCCTGAAGGTAAAGACAAA
 TTTGTATTTACTGGAGGCCGTGGTGGTGCTGGGCATGCTATGGTCACCGTTGCTTCAGATATCACGGAAGCCCGC
 CAAAGGATACTGGAGCTGTTAGAGCCCAAAGGGACCGGGGAGTCCAAAGGTGCTGGGGAGTCAAAGGCGTTGGG
 GAGTTGAGGGAGTCAAATAGCGGTGCGGAAAACACCACAGAACTCAGACCTCAACCTCAACTTCCAGCCTTCGT
 TCAGATCCTAAACTTTGGTTGGCGTTGGGGACTGTTGCTACAGGTCTGATAGGGTTGGCGGCGACGGGTATTGTA
 CAGGCGCTTGCAATTGACGCCGGAGCCGGATAGCCCAACCACGACCGACCTGATGCAGCTGCAAGTGCAACTGAA
 ACTGCGACAAGAGATCAGTTAACGAAAGAAGCGTTCCAGAACCAGATAATCAAAAAGTTAATATCGATGAGCTC

FIGURE 6A

GGAAATGCGATTCCGTCAGGGGTATTGAAAGATGATGTTGTTGCGAATATAGAAGAGCAGGCTAAAGCAGCAGGC
 GAAGAGGCCAAACAGCAAGCCATTGAAAATAATGCTCAGGCGCAAAAAAATATGATGAACAACAAGCTAAACGC
 CAGGAGGAGCTGAAAGTTTCATCGGGGGCTGGCTACGGTCTTAGTGGCGCATTGATTCTTGGTGGGGGAATTGGT
 GTTGCCGTCACCGCTGCGCTTCATCGAAAAAATCAGCCGGTAGAACAAACAACAACAACAACACTACTACA
 ACTACAAGCGCACGTACGGTAGAGAATAAGCCTGCAAATAATACACCTGCACAGGGCAATGTAGATACCCCTGGG
 TCAGAAGATACCATGGAGAGCAGACGTAGCTCGATGGCTAGCACCTCGTCGACTTTCTTTGACACTTCCAGCATA
 GGGACCGTGCAGAATCCGTATGCTGATGTTAAAACATCGCTGCATGATTCGCAGGTGCCGACTTCTAATTCTAAT
 ACGTCTGTTTCAAGATATGGGGAATACAGATTCTGTTGTATATAGCACCATTCAACATCCTCCCCGGGATACTACT
 GATAACGGCGCACGGTTATTAGGAAATCCAAGTGCGGGGATTCAAAGCACTTATGCGCGTCTGGCGCTAAGTGGT
 GGATTACGCCATGACATGGGAGGATTAACGGGGGGGAGTAATAGCGCTGTGAATACTTCGAATAACCCACCAGCG
 CCGGGATCCCATCGTTTTCGTCGGTCTGCTCCACCGGTGAAAGTGCAGACAAGAGATCAGTTAACGCAAGAAGCA
 TTCAAGAATCCTGAGAACCAGAAAGTTAGCATTGATGAGATCGGAAATTCTATTCCGTCTGGGGAATTAAAAGAT
 GATGTTGTTGCTAAAATAGAAGAACAAGCTAAAGAGGCGGGTGAGGCGGCCAGACAGCAGGCTGTTGAAAGCAAT
 GCACAGGCGCAGCAGCGATATGATACTCAGTATGCCAGACGTCAGGAGGAATTATCAGGATCGGGGACTAGTGCA
 CAGGCTGTTGCGTTGACTCCAGAGCCGGATGACCCAATCACTACCGACCCTGATGCTGCAGCAAACACAGCTGAA
 GCAGCGGCAAAAGATCAGTTAACGAAAGAAGCATTCCAGAACCCAGATAACCAGAAAGTTAATATCGATGAGAAC
 GGAAATGCAATTTTCATCCGGCGGAATGCATGGGGAACAGGCCAGGAGCTATTGAAAGTAATTCTCAGGCG
 CAGAAAAAATATGATGAGCAGCATGCTAAACGCGAACAGGAAATGTAA

FIGURE 6A (CONT'D)

1 MATVIDLSFPKTGAKKIILYIPQNYQYDTEQGNGLQDLVKAAEELGIEVQREERNNIATA
 61 QTSLGTIQTAIGLTERGIVLSAPQIDKLLQKTKAGQALGSAESIVQNANKAKTVLSGIQS
 121 ILGSVLGMDLDEALQNNNSQHALAKAGLELTNSLIENIANSVKTLDEFGEQISQFGSKL
 181 QNIKGLGTLGDKLKNIGGLDKAGLGLDVISGLLSGATAALVLADKNASTAKKVGAGFELA
 241 NQVVGNIITKAVSSYILAQRVAAGLSSTGPVAALIASTVSLAISPLAFAGIADKFNHAKSL
 301 ESYAERFKKLGVDGDNLLAEYQRGTTIDASVTAIN TALAAIAGGVSAAGSVIASPIA
 361 LLVSGITGVISTILQYSKQAMFEHVANKIHNKIVEWEKNNHGKNYFENGYDARYLANLQD
 421 NMKFLNLNKELQAERVIAITQQQWDNNIGDLAGISRLGEKVLSGKAYVDAFEEGKHKA
 481 DKLVQLDSANGIIDVSNNGKAKTQHILFRTPLLTPGTEHRERVQTGKYEYITKLNINRVD
 541 SWKITDGAASSTFDLTNVVQRIGIELDNAGNVTKTKETKIIAKLGEEDDNV FVGS GTTEI
 601 DGGEGYDRVHYSRGNYGALTIDATKETEQQSYTVNRFVETGKALHEVTSTHTALVGNREE
 661 KIEYRHSNNQHHAGYYTKDTLKAVEEIIIGTSHNDIFKGSKFND AFNGGDGVD TIDGNDGN
 721 DRLFGGKGDDILDGGNGDDFIDGGKGNLLHGGKGDDIFVHRKGDGNDIITDSDGNDKLS
 781 FSDSNLKD LTFEKVKHNLVITNSKKEKVTIQNW FREADFAKEVPNYKATKDEKIEEIIIGQ
 841 NGERITSKQVDDLIAGNGKITQDELSKVVDNYELLKHSKNVTNSLDKLISSVSAFTSSN
 901 DSRNVLVAPTSMLDQSLSSLQFARGSPIGNLGHNPVNNSIPPAPPLPSQTDGAGGRGQL
 961 INSTGPLGSRALFTPVRNSMADSGDNRASDV PGLPVNPMRLAASEITLNDGFEVLHDHGP
 1021 LDTLNRQIGSSVFRVETQEDGKHIAVGQRNGVETSVVLS DQEYARLQSIDPEGKDKFVFT
 1081 GGRGGAGHAMVTVASDITEARQRIELLEPKGTGESKGAGESKGVGELRESNSGAENTTE
 1141 TQTSTSTSSLRSDPKLWLALGTVATGLIGLAATGIVQALALTPEPDSPTT TDPDAAASAT
 1201 ETATRDQLTKEAFQNP DNQKVNIDELGNAIPSGVLKDDVVANIEEQAKAAGEEAKQQAIE
 1261 NNAQAQKKYDEQQAQRQEELKVSSGAGYGLSGALILGGGIGVAVTAALHRKNQPVEQTTT
 1321 TTTTTTTTTSARTVENKPANNTPAQGNVDTPGSED TMESRRSSMASTSSTFFDTSSIGTVQ
 1381 NPYADVKTSLHDSQVPTSNSNTSVQNMGN TDSVVYSTIQHPPRD TTDNGARLLGNPSAGI
 1441 QSTYARLALSGGLRHD MGGLTGGSNSAVNTSNNPPAPGSHRFVSGSGSTGESATRDQLTQE
 1501 AFKNPENQKVSIDEIGNSIPSGELKDDVVAKIEEQAKEAGEAARQQAVESNAQAQQRYDT
 1561 QYARRQEELSGSGTSAQAVALTPEPDDPIT TDPDAAANTAEAAAKDQLTKEAFQNP DNQK
 1621 VNIDENGNAISSGGMHGEQARQEAI ESNSQAQKKYDEQHAKREQEM

FIGURE 6B

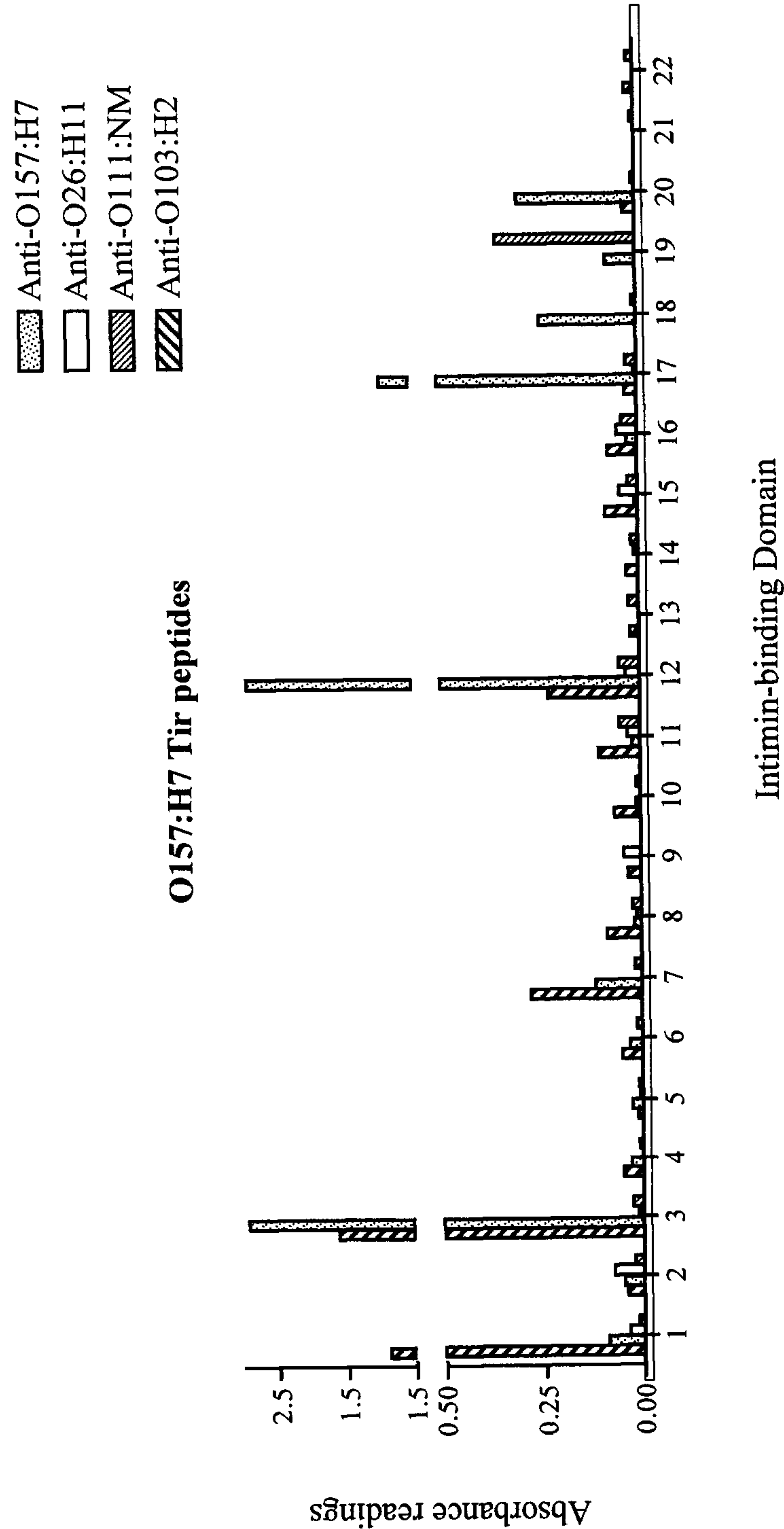


Fig. 7

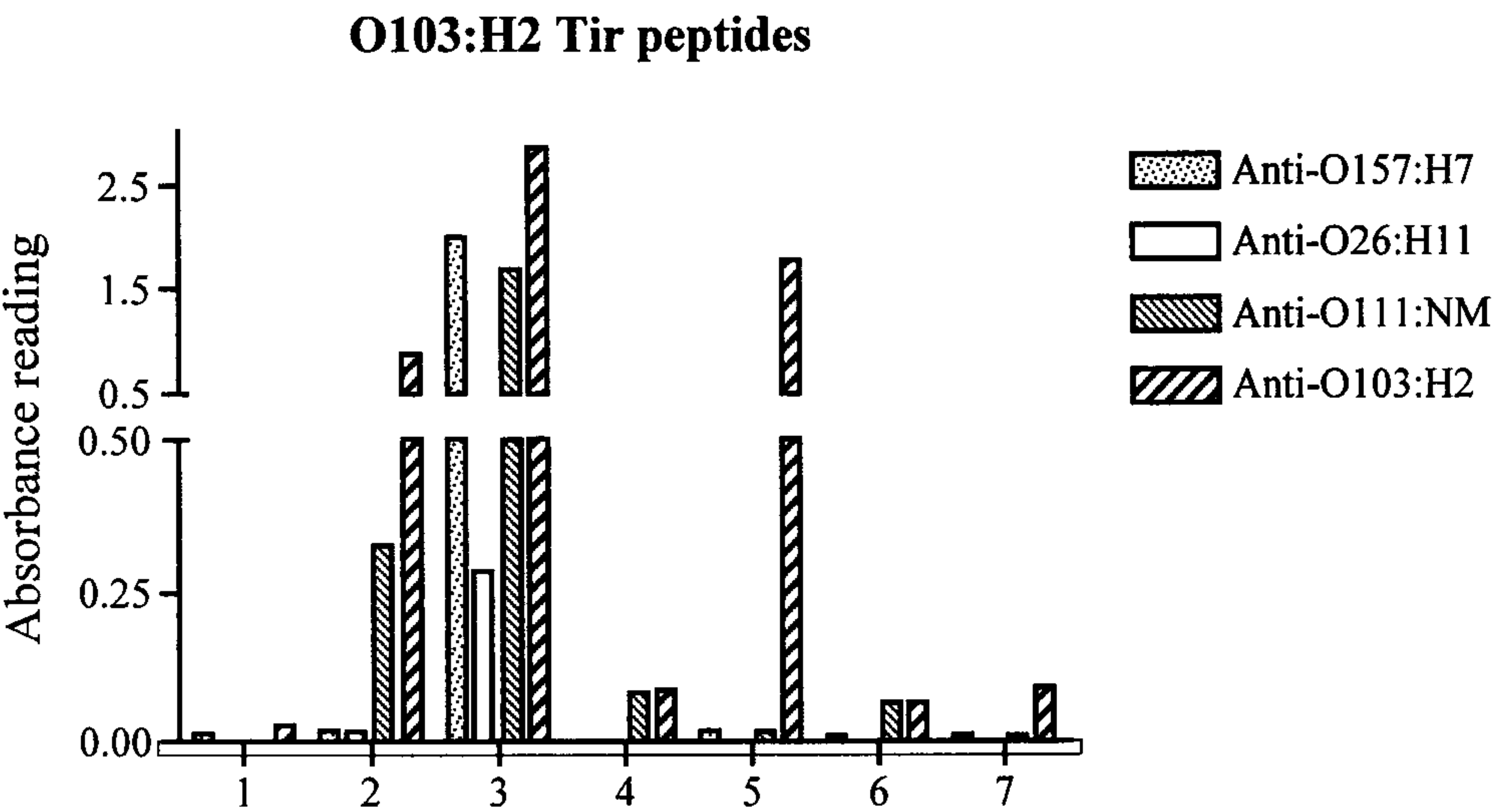


Fig. 8A

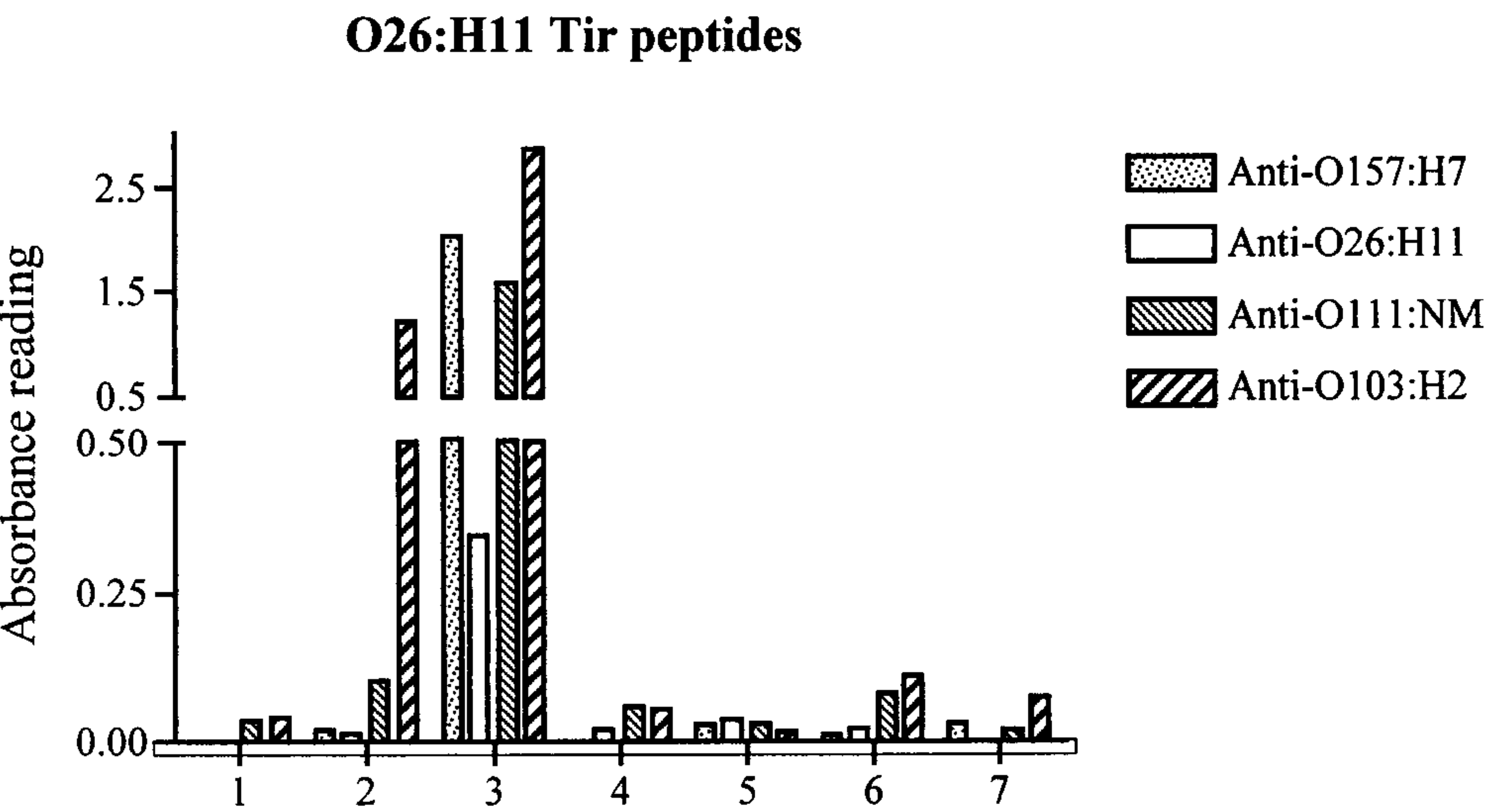


Fig. 8B

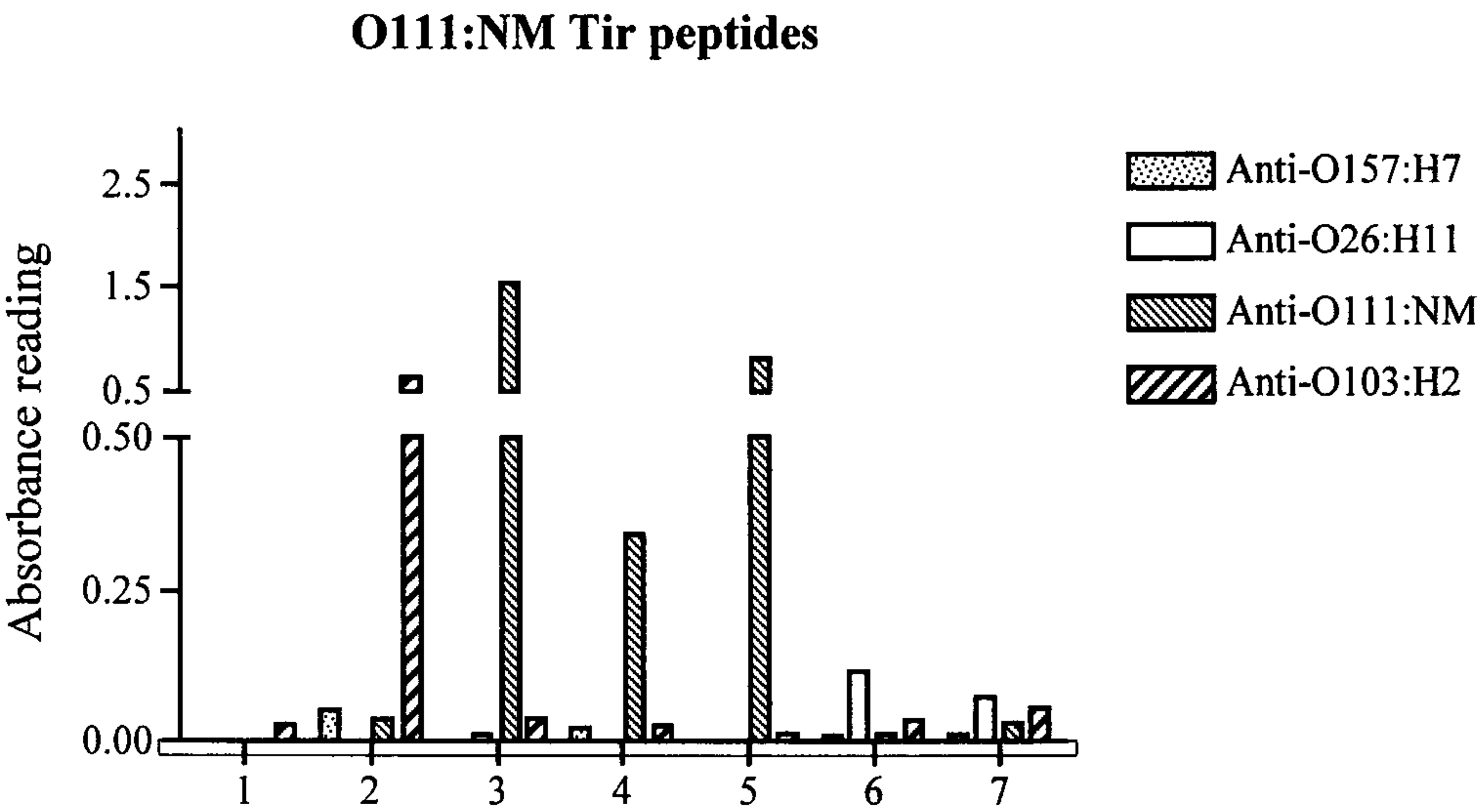


Fig. 8C

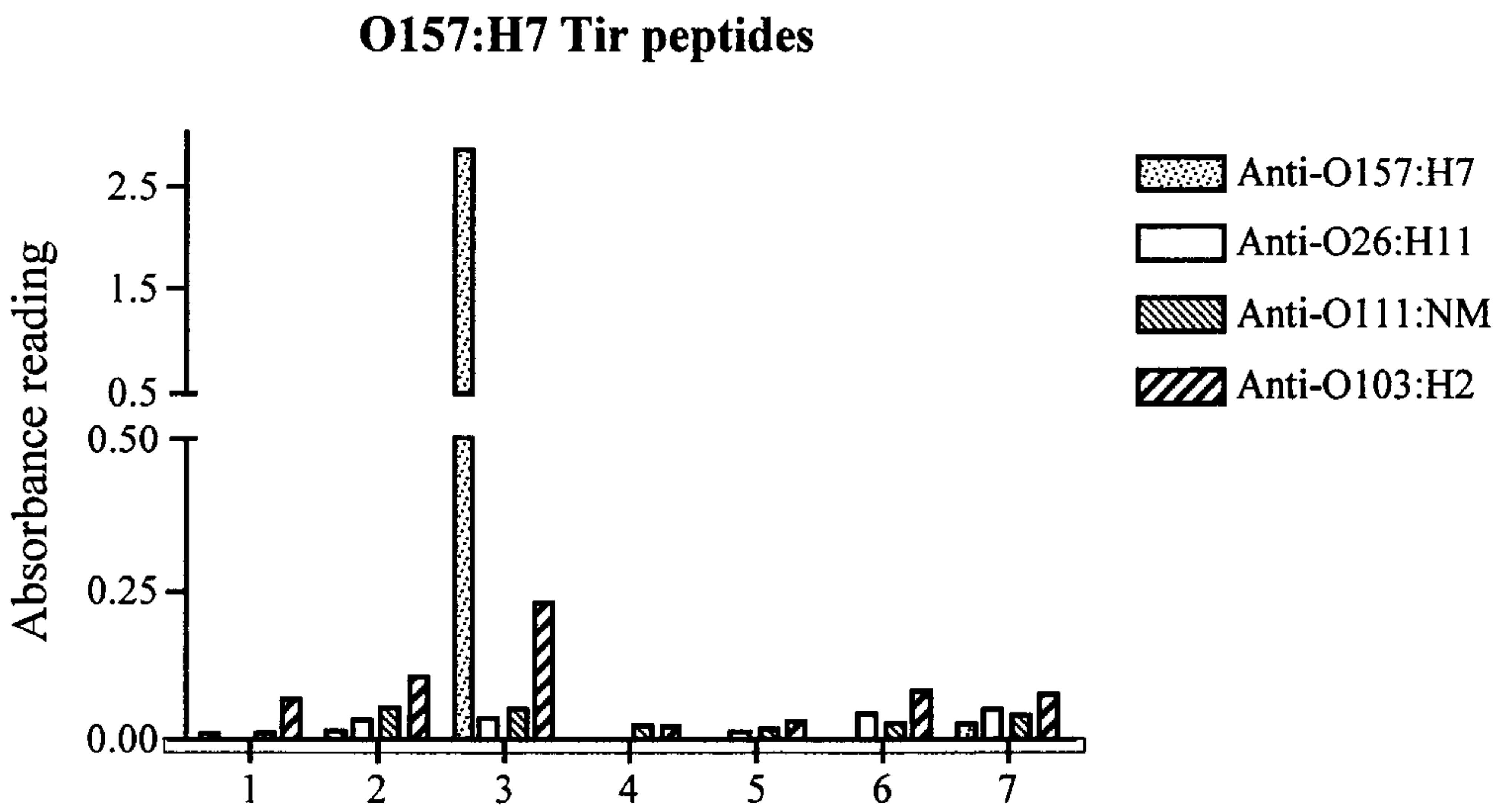


Fig. 8D

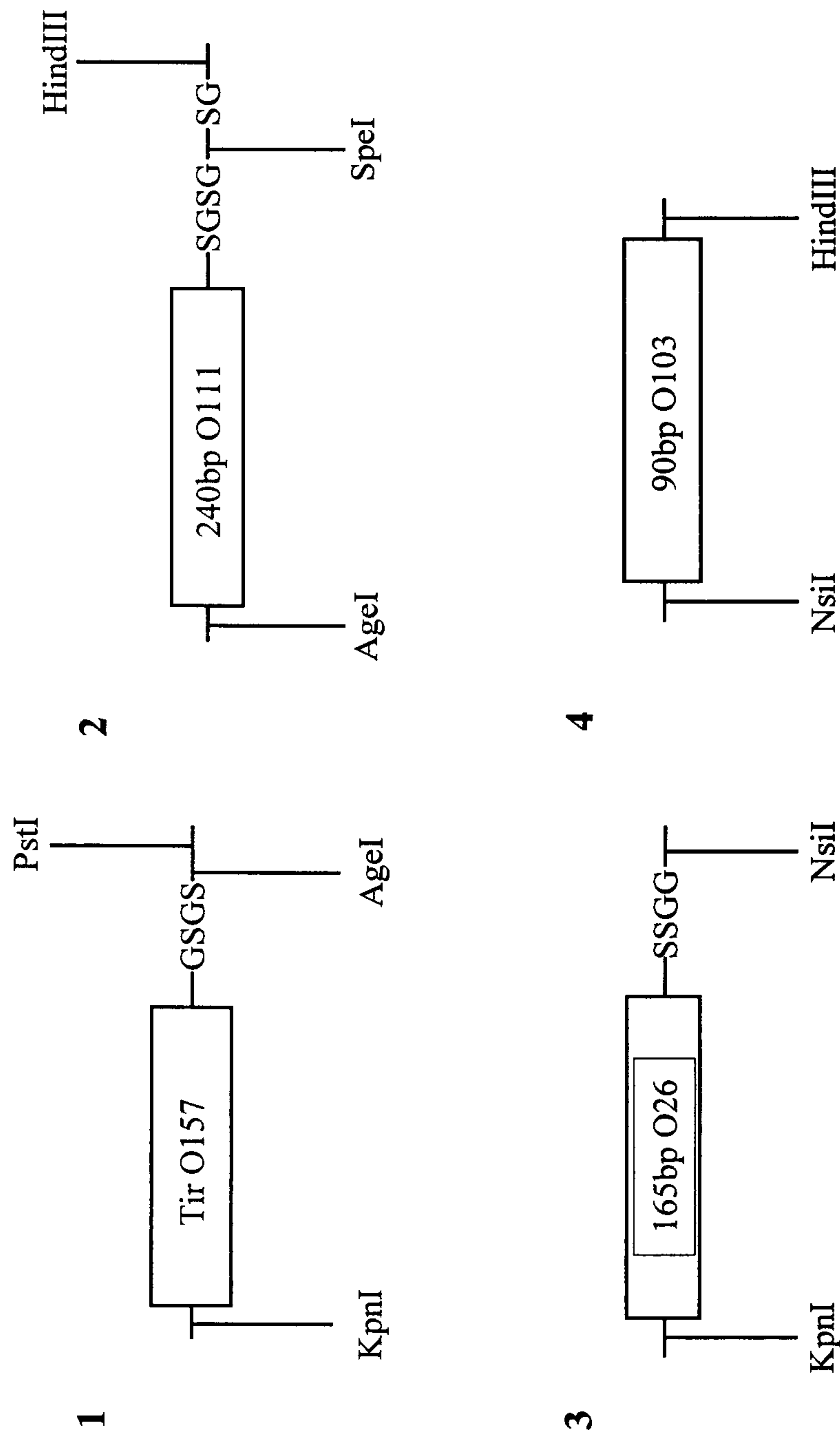


Fig. 9A



Fig. 9B



↑
Fused to
Leukotoxin

Fig. 9C

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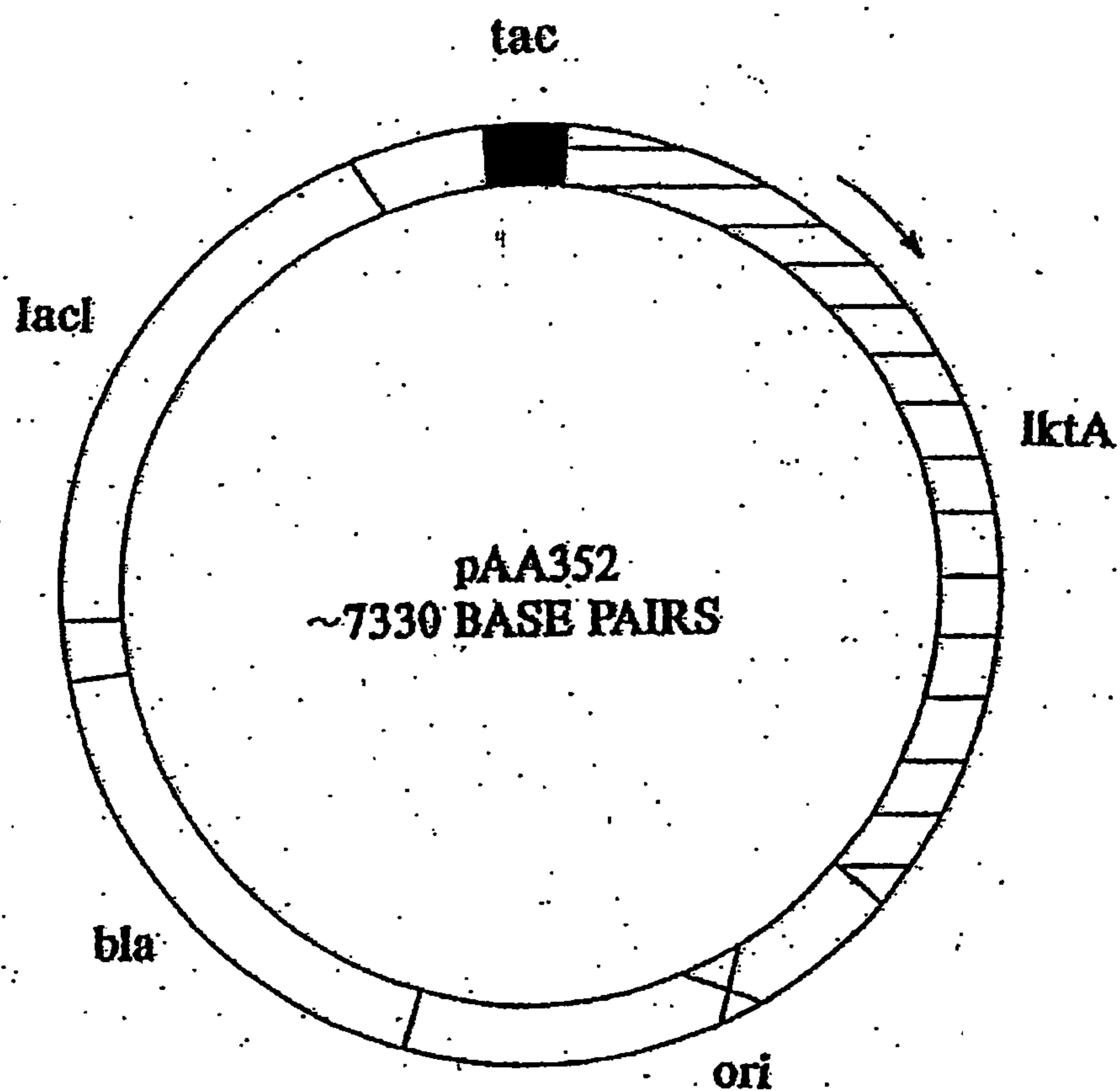


FIG. 10

FIG. 11A

```

280  AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT GCG GAA AGC ATT GTA CAA AAT AAA GCG AAA ACT GTA TTA TCT GGC ATT CAA TCT
290  TTT TGA TTT CGT CCG GTT CGT AAT ECA AGA CCG CTT TCG TAA CAT Val Ile Val Val Val Val Val Val Val Val Val Val Val
300  Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala RECOMBINANT LEUKOTOXIN PEPTIDE C
310  320  330  340  350  360
370  ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG
380  TAA AAT CCG AGT CAT AAC CGA CCT TAC CTA AAT TTA AAT CTA CTC CGG AAT GTC TTA TTG TCG TTG GTT GTA CGA GAA CAT TTT CGA CCG AAC CTC
390  Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp Asp Leu Asp Asp Leu Asp Asp Leu Asp Asp Leu Asp Asp Leu Asp Asp Leu
400  410  420  430  440  450
460  CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA CTT GAG GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
470  GAT TGT TTA AGT AAT TAA CTT TTA TAA CGA TTA AGT CAT TTT TGT GAA CTG CTT AAA CCA CTT Gln Gln Ile Ser Gln Phe Gly Ser Lys Leu
480  Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Gln Gln His Ala Leu Ala Lys Ala Gly Leu Glu
490  500  510  520  530  540
550  CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA
560  GAT TTA TAG TTT CCG AAT CCC TGA AAT CCT CCG TCG TTT GAG TTT GAG TTT TTA TAG CCA CCT GAA CCG GAA CCA AAT CTA CAA TAG AGT
570  Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Asp Val Ile Ser
580  590  600  610  620  630

```

FIG. 11B

640 * TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
650 * GAT AAT AGC CCG CGT TGT TGA CGT CTA TTT TTA CGA AGT TGT CGA TTT TTT CAC CCA CGC CCA AAA TTT GAA CTT AAC CGT
660 * Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala
670 * GCA GAT AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA CTT AAC CGT
680 * TTA CGA AGT TGT CGA TTT TTA CGA AGT TGT CGA TTT TTT CAC CCA CGC CCA AAA TTT GAA CTT AAC CGT
690 * ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA CTT AAC CGT
700 * TTT TTT CAC CCA CGC CCA AAA TTT GAA CTT AAC CGT
710 * TTT TTT CAC CCA CGC CCA AAA TTT GAA CTT AAC CGT
720 * TTT TTT CAC CCA CGC CCA AAA TTT GAA CTT AAC CGT
730 * GGT AAT ATT ACC AAA GCC GGT TTT CCA GGT TTT TCA ACT GCG CCT GTG
740 * TTA TAA TTT TTT CCA GGT TTT TCA ACT GCG CCT GTG
750 * TTT CCG CAA Val Ser Ser Tyr Leu Ala Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val
760 * TCT TCT TAC ATT TTA GCC CAA CGT GGT GCA GGT TTT TCA ACT GCG CCT GTG
770 * TTA AAT GCT TTT GCA GGT TTT TCA ACT GCG CCT GTG
780 * TTT GCA GGT TTT TCA ACT GCG CCT GTG
790 * TTT GCA GGT TTT TCA ACT GCG CCT GTG
800 * TTT GCA GGT TTT TCA ACT GCG CCT GTG
810 * TTT GCA GGT TTT TCA ACT GCG CCT GTG
820 * GCT TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
830 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
840 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
850 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
860 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
870 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
880 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
890 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA
900 * TTA ATT GCT TCT ACT TGA CAA GAT TTT GCG GAT AAA TTT AAT CAT GCA AAA AGT TTA

FIG. 11C

[illegible]

[illegible]

FIG. 11F

FIG. 11G

FIG. 11H

[illegible]

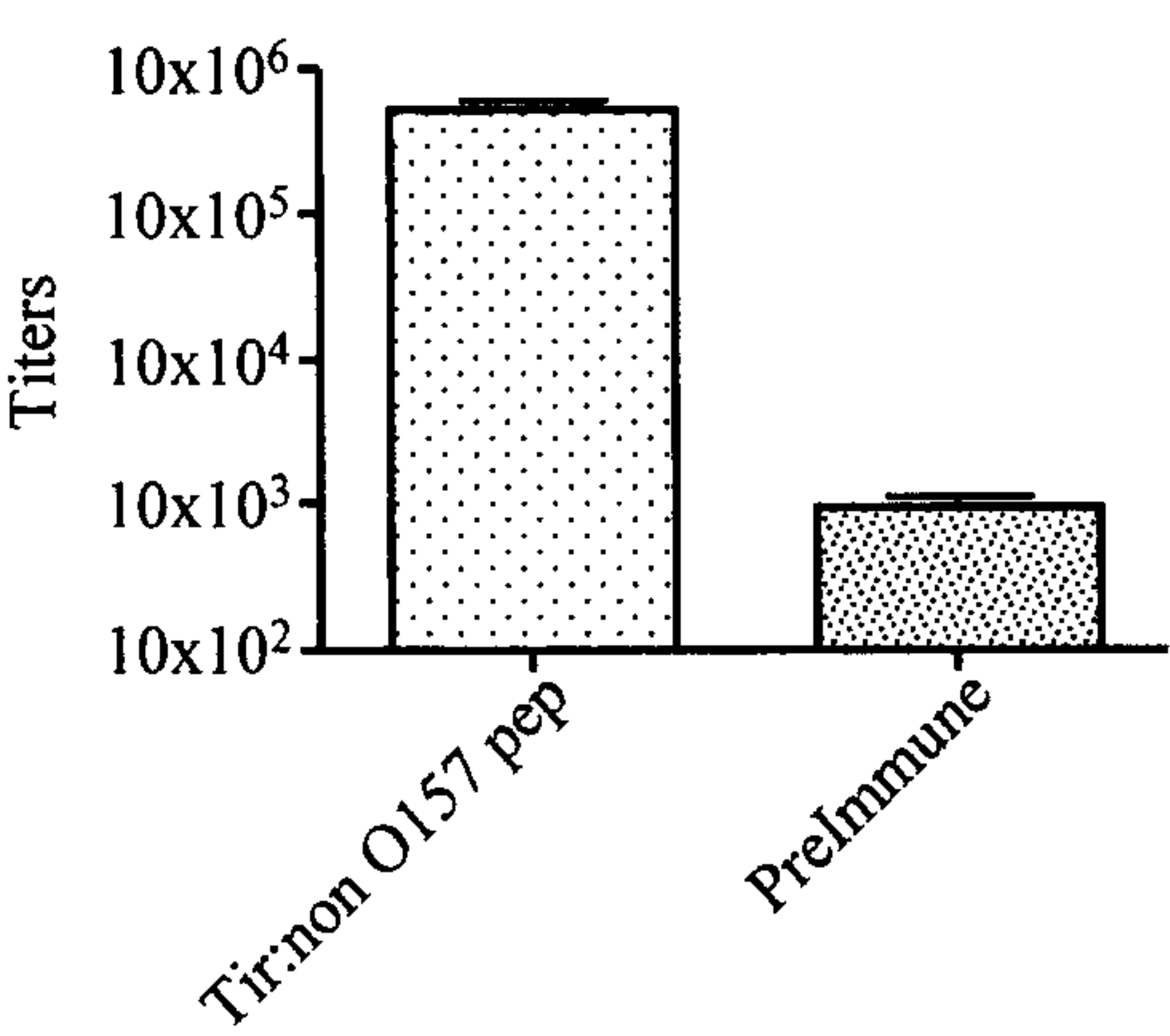


Fig. 12A

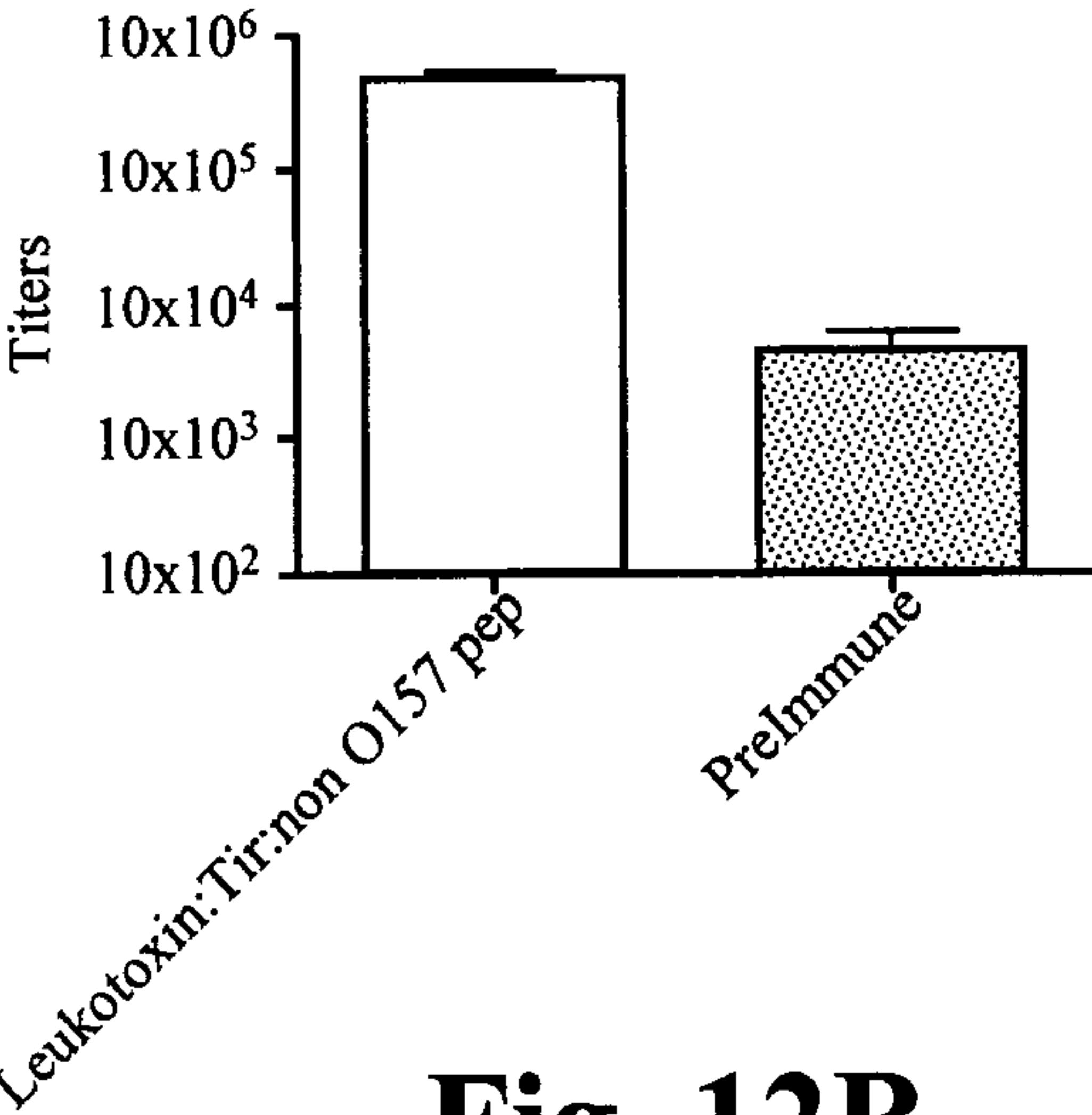


Fig. 12B

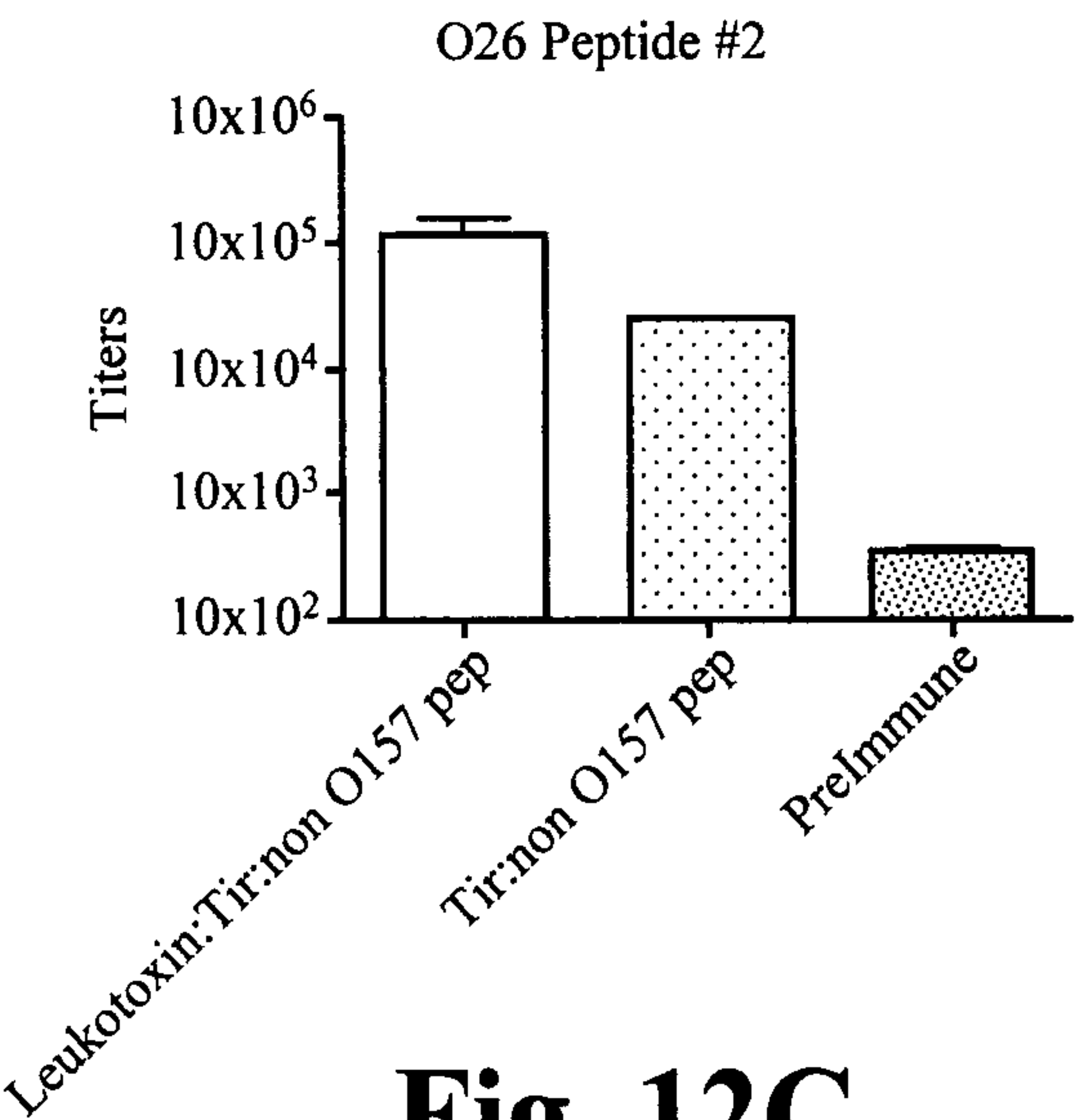


Fig. 12C

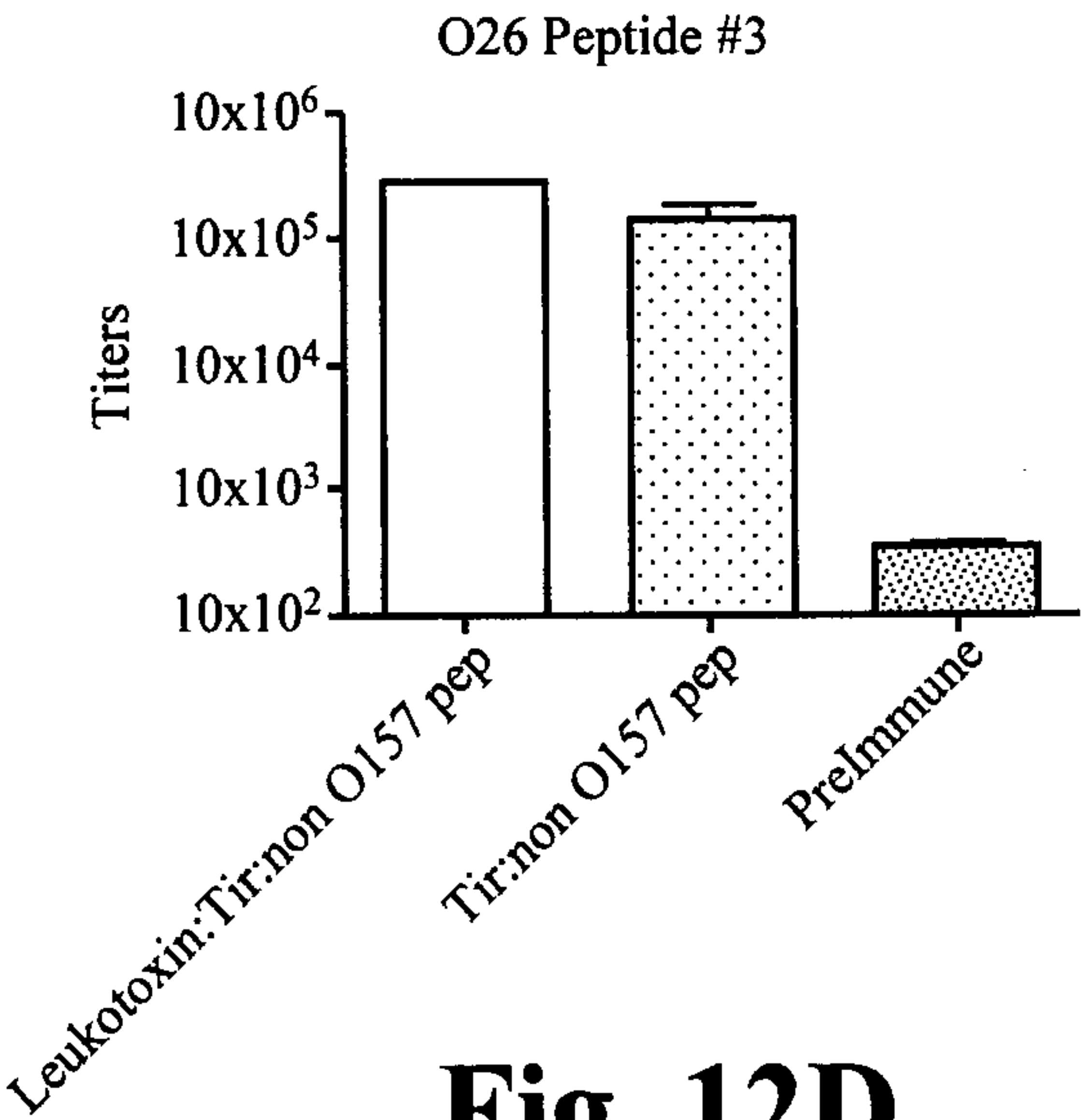


Fig. 12D

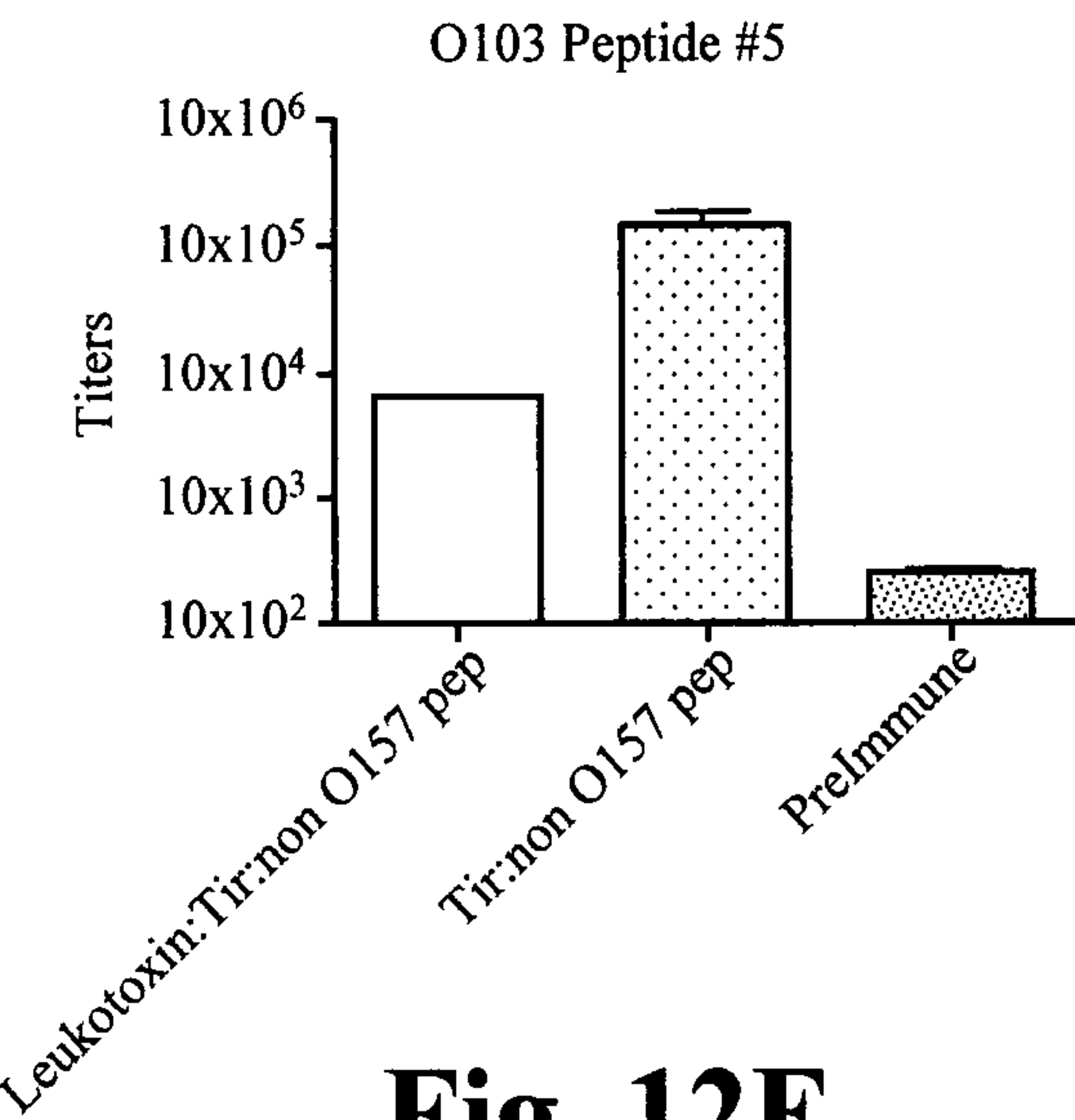


Fig. 12E

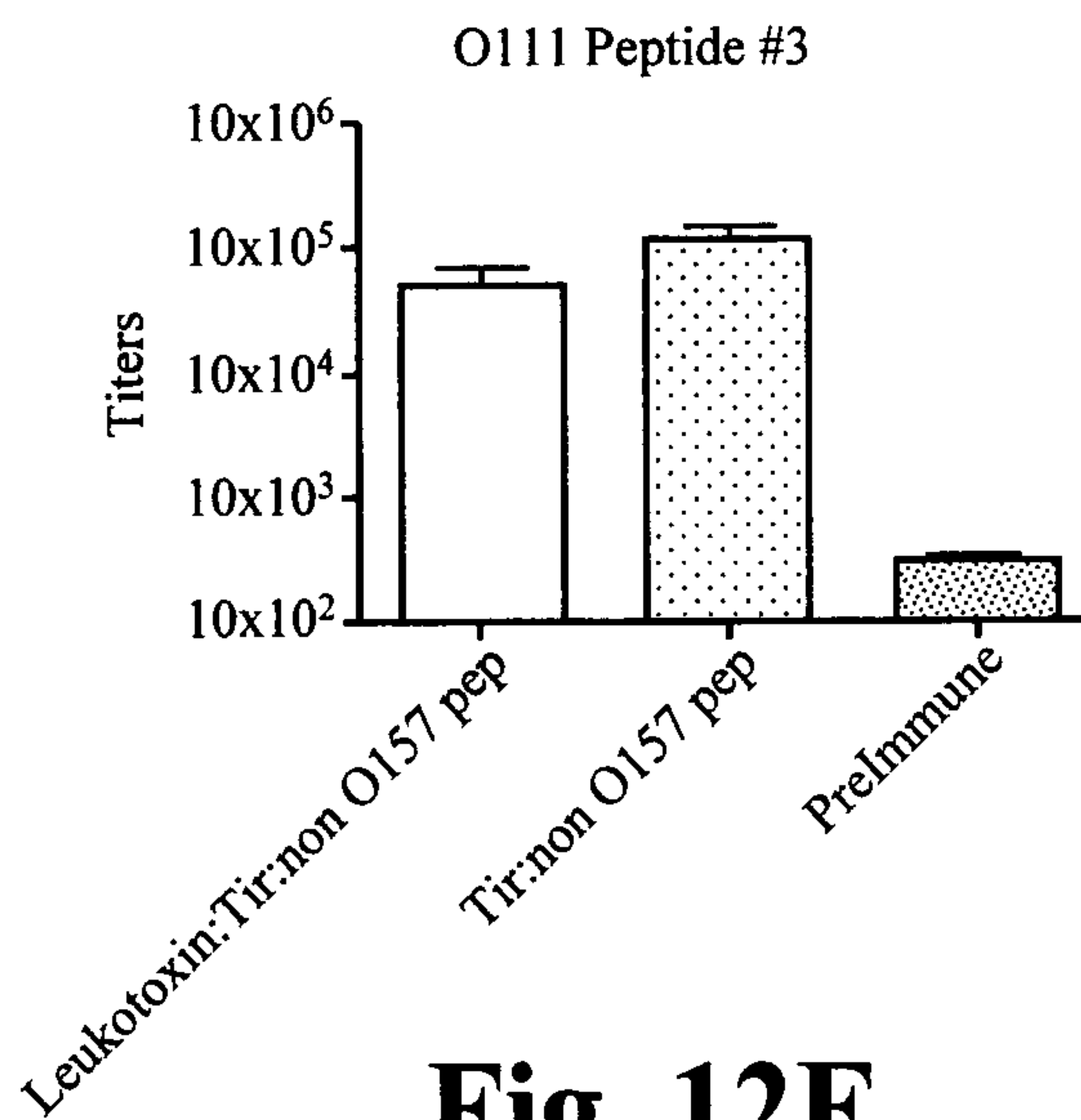


Fig. 12F

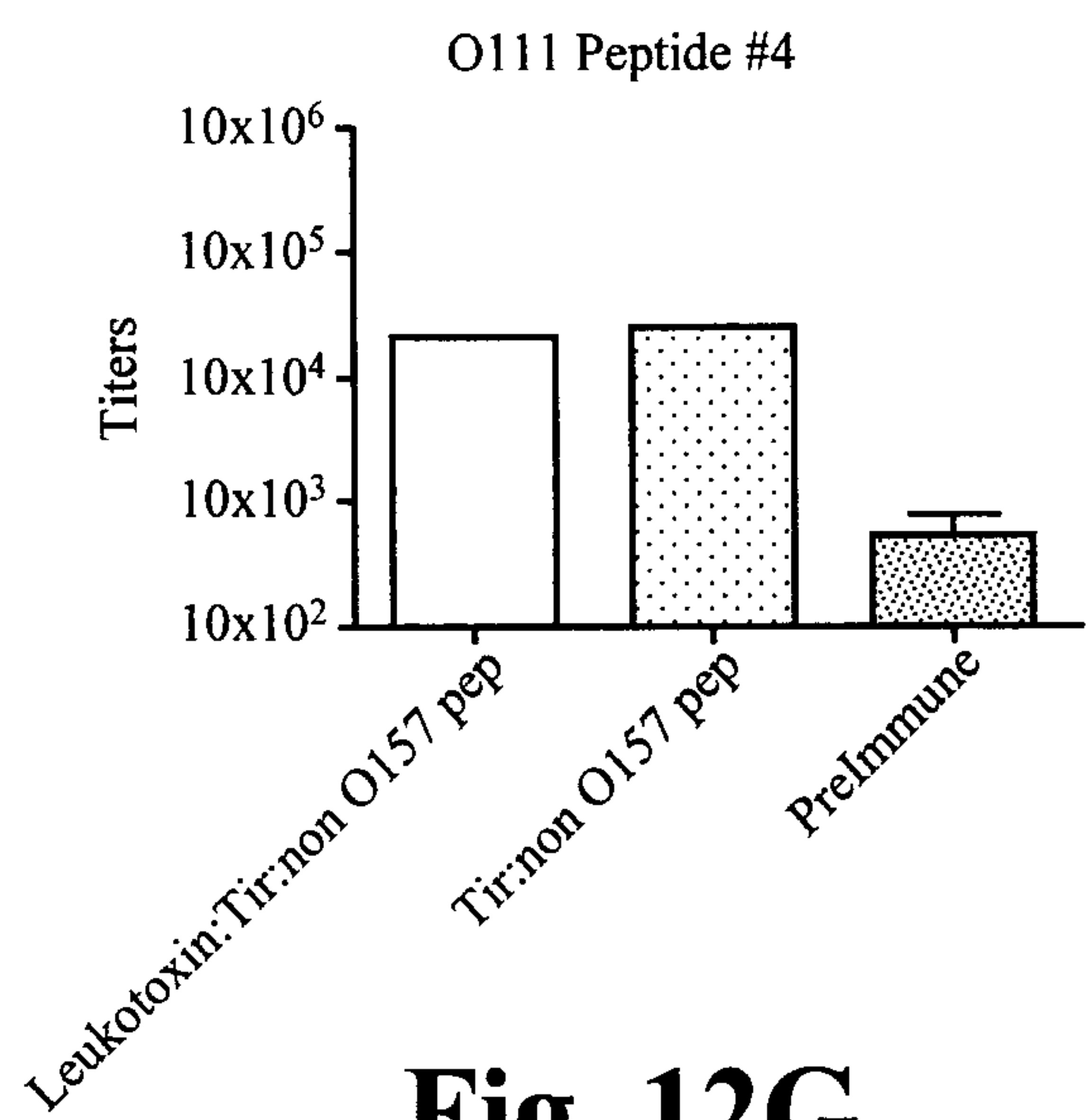


Fig. 12G

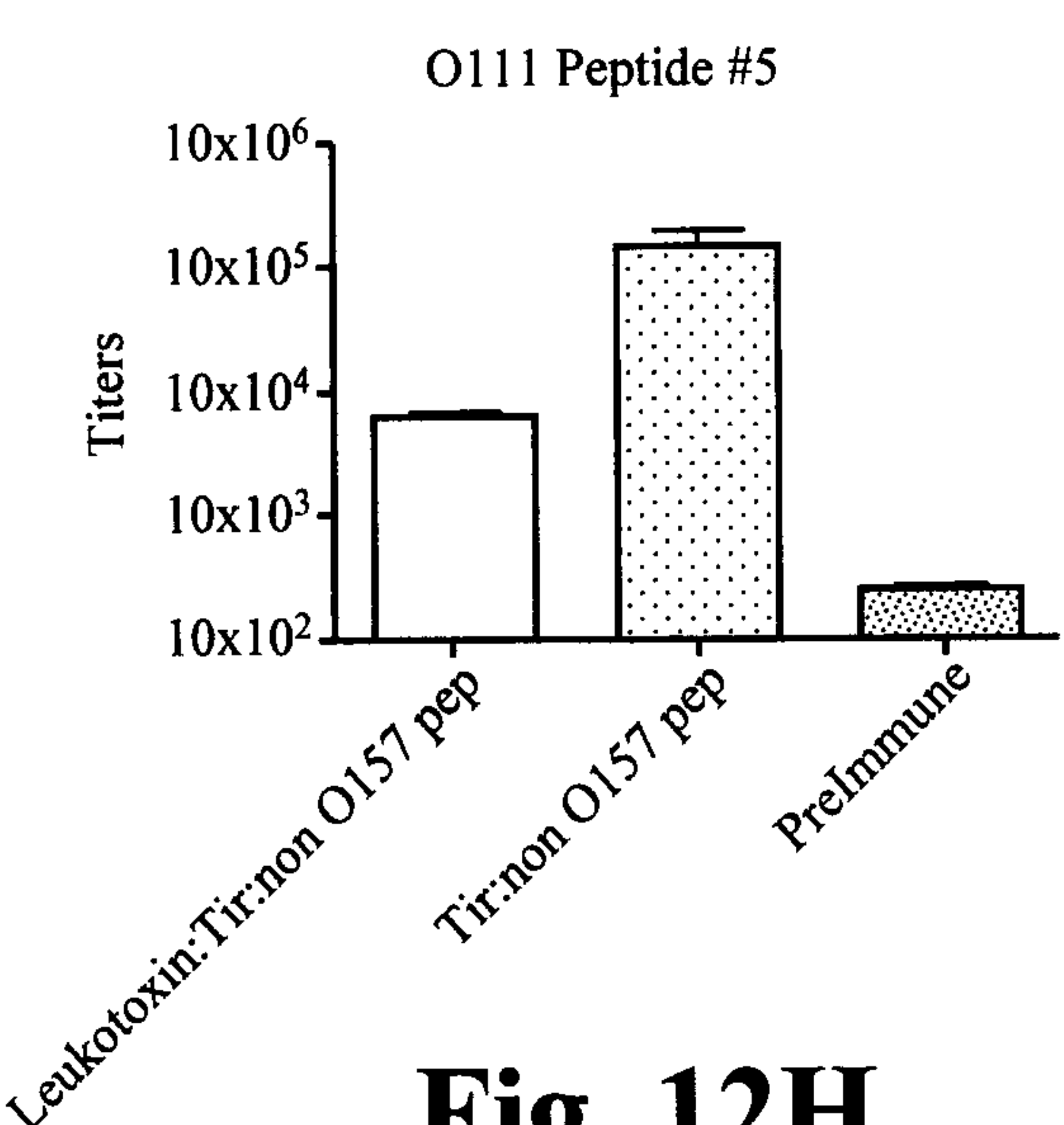


Fig. 12H

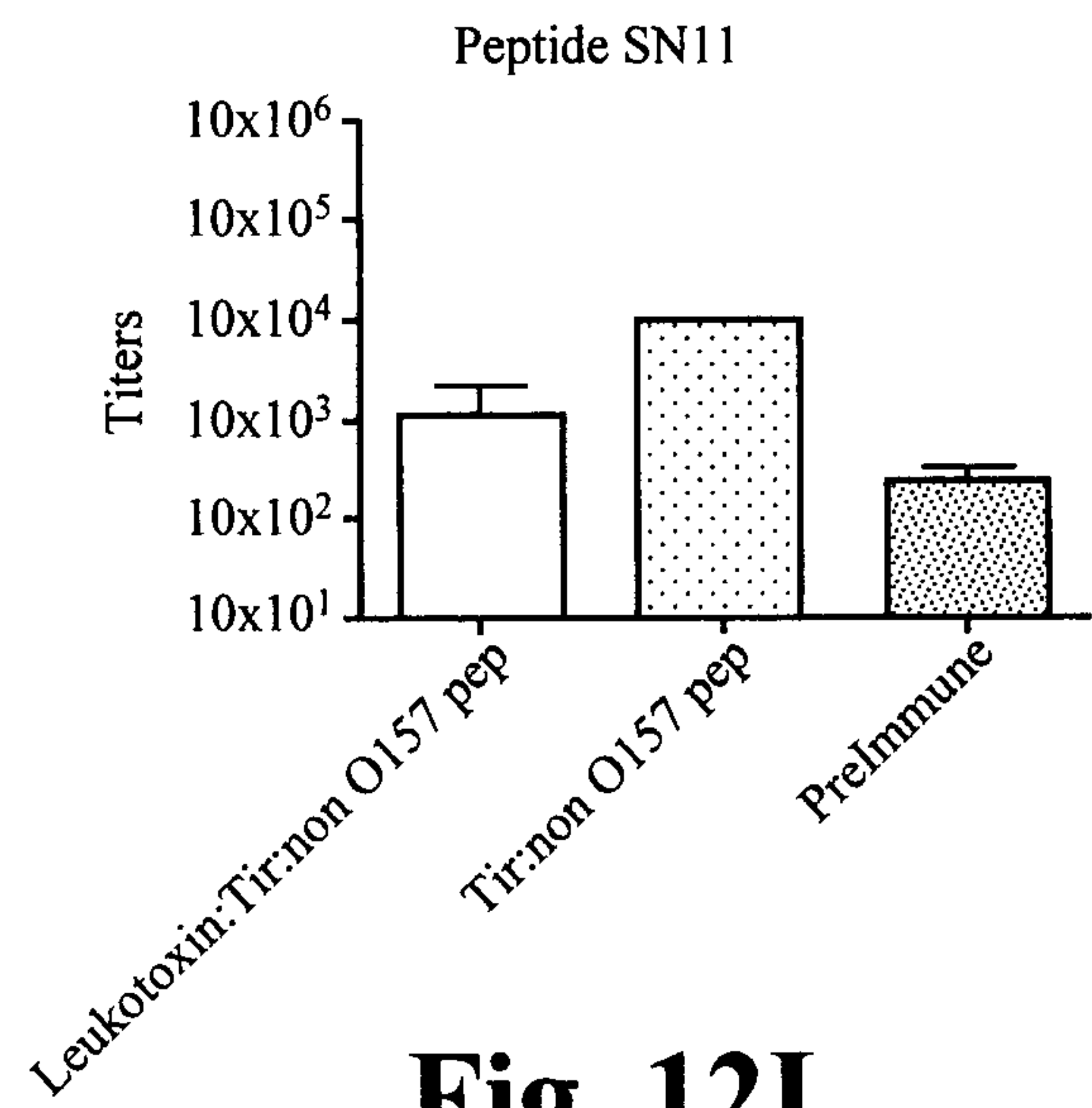


Fig. 12I

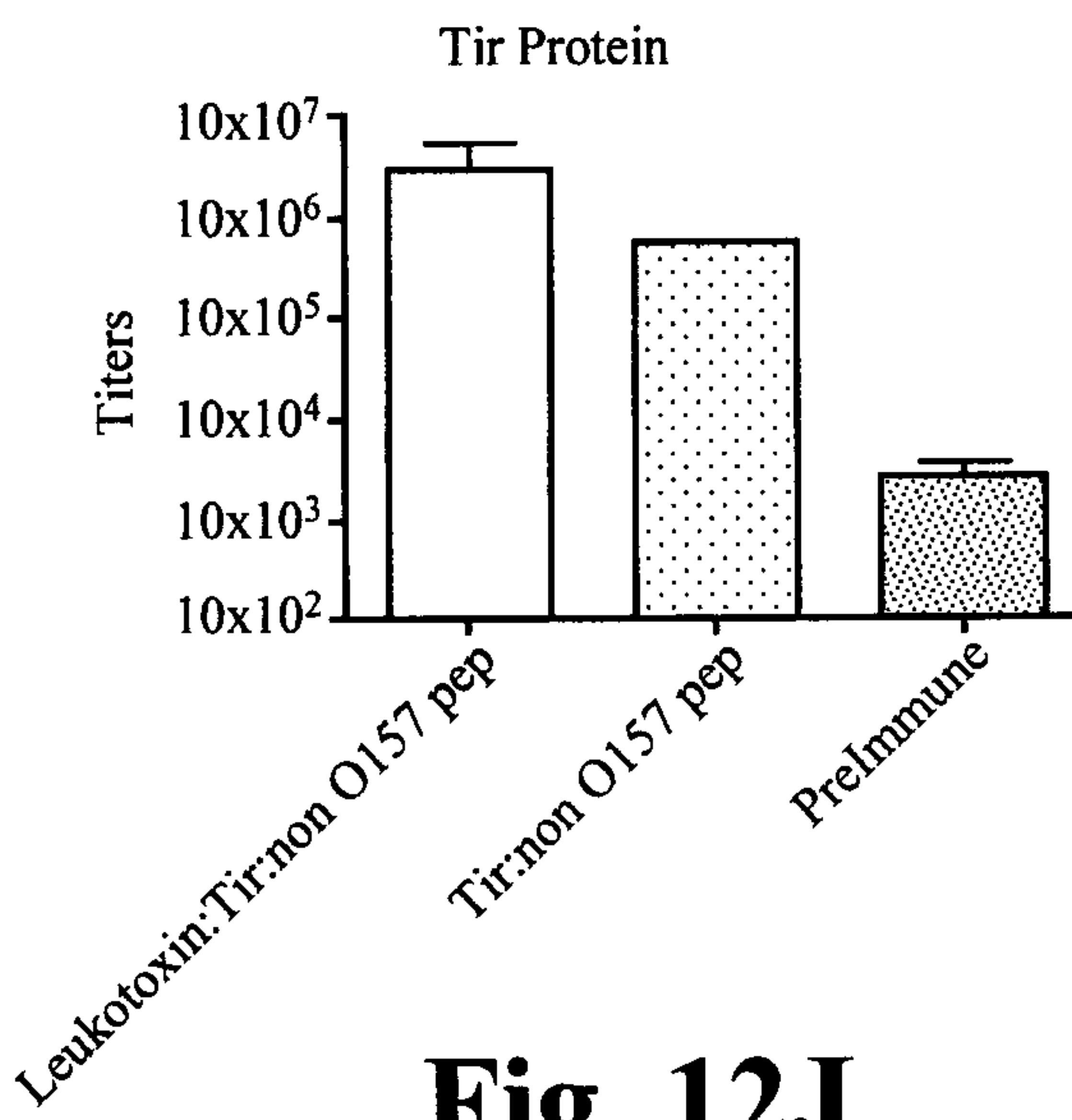
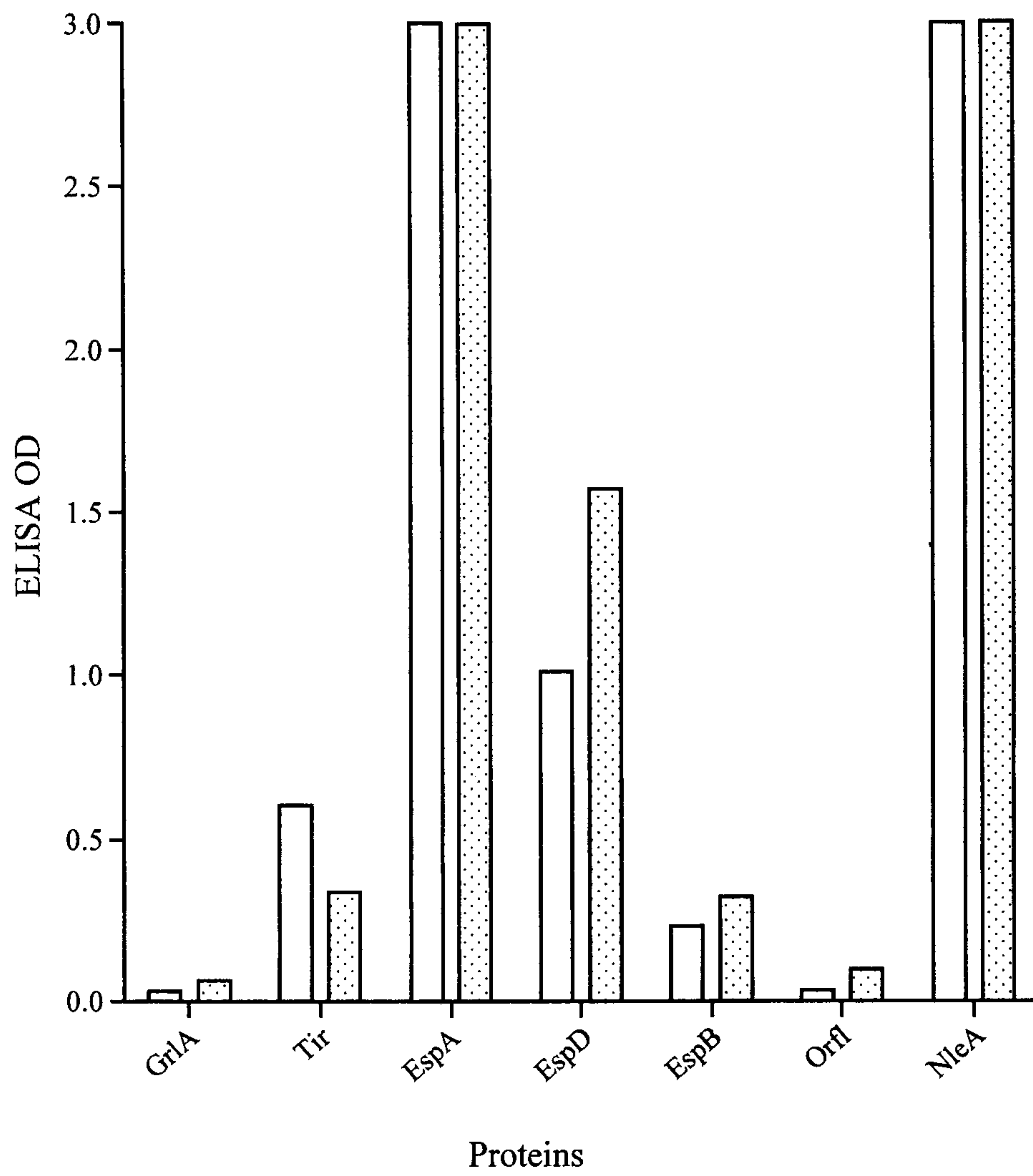


Fig. 12J

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**Fig. 13**

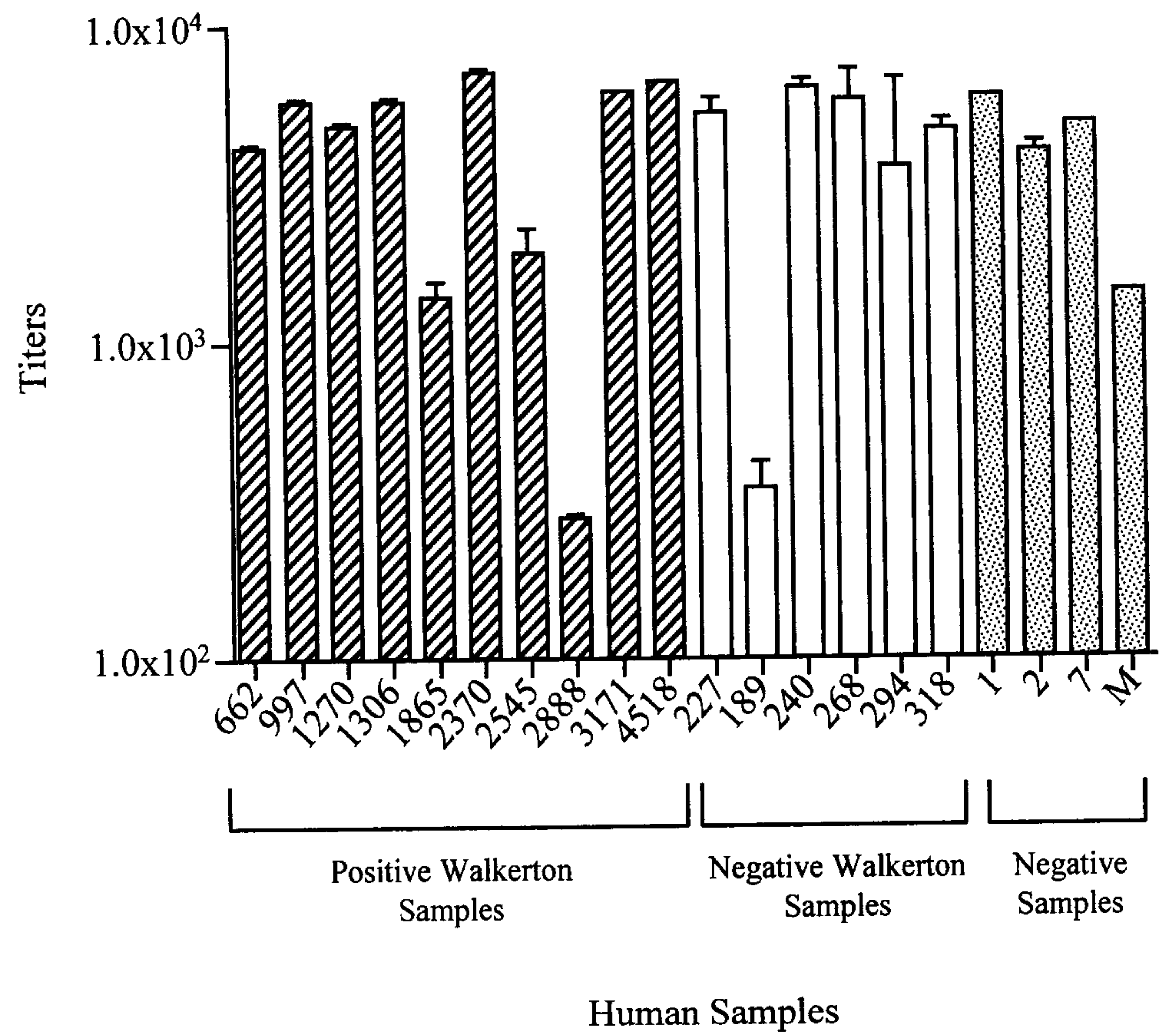


Fig. 14

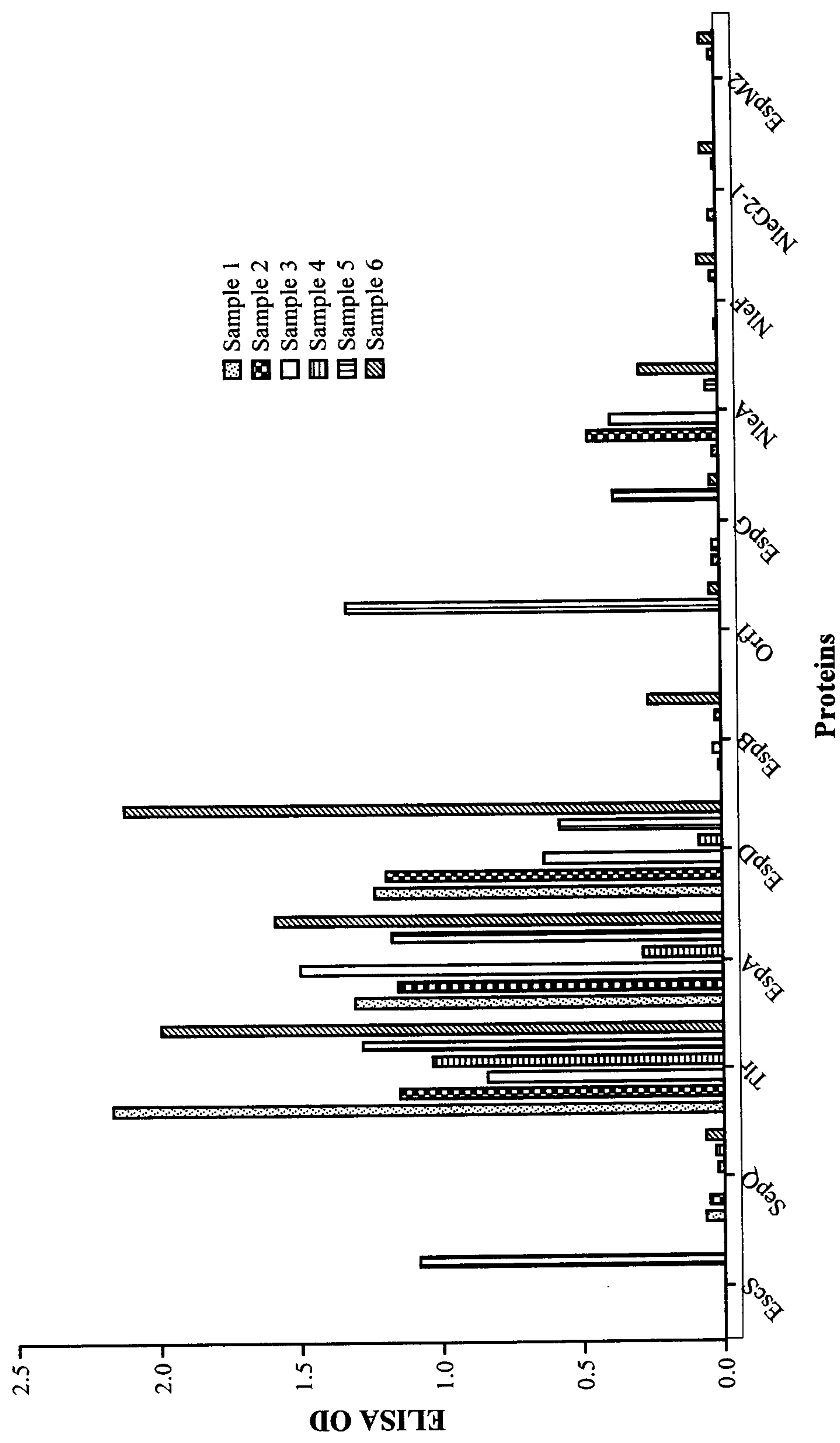


Fig. 15

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```
1  ttatattacca agggatattg ctgaaatagt tctatattgt agagattgca catcagaacg
61  tgcactcggtt aagagattta atgtattcga catttgctga atttcgagct ggctattatt
121 cactaccggtt gtcagggttat tcgcttttagc tgaaatagcc gccttcactg tttgcagatc
181 accagcgctt aaatcaccac taagatcacg aataccagtt acacttatgt cattacgtgg
241 atcgttttata tagtcaatca cgtcttgagg aagtttggct ttcgcattct tatcagtgct
301 actctgaaca tcagcaattht tggcatccac aagattagcc atctttttgtg ccgtgggttga
361 cgcttttagat gcctcattca tatcagcaaa ctttgcaatc gacagattac tttgtgcctg
421 atacatataa gaaaacatga gaatcgcagc ctgaaaaaca ccgagttcct caaatagctt
481 aaccacctca tccttcgaca tattacctaa gtcatagatc gtcgatgtcg aagaactcgc
541 actcacatta acaacggatg ttgcatttga tgtatccat
```

A

```
1  MDTSNATSVVNVSSASSSTSTIYDLGNMSKDEVVKLFEEELGVFQAAILMFS
51  YMYQAQSNLSIAKFADMNEASKASTTAQKMANLVDAAKIADVQSSTDKNK
101 AKLPQDVIDYINDPRNDISVTGIRDLSGDLQTVKAAISAKANNLT
151 TVVNNSQLEIQQMSNTLNLLTSARSDVQSLQYRTISAIKLGK
```

B**FIGURE 16**

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```

1  ttacccagct aagcgacccg attgccccat acgattctgg acctcaagga gatcgcgga
61  agcggacgtt atatcacgca gacgagtcgt gatatcatcc tgcgttctgc gaacgtcttg
121 tttatacagc tccagattcc cctgctggaa gttttccagc gacttcgcac gttgttcatt
181 aacctcatga gtcgatttga cggactcaga tattgttggt ggcaacgttt tcgtaccttc
241 agcaagactg gtcacggcaa caaatgctgt ggtattgggt agtttatcta cggaattcaa
301 caacttattg attctgcttg tcttctcggc ggctctgca agatcttcag caaagtcaga
361 ggctttcgca acatcatctg caacgccaga tgcacggctg gctgctttcg ttgttggtgc
421 catcgctttc tgcacgcac tggatgcctc ctctgcgaca tcagcaacac tttccgtagc
481 cttgaccaga gctttatttg caacctcaga agccgcacca gcagcctttg aagatgcaga
541 gcttgctttt tcagcaatct caccagcccc tttagccgcg ttgttcattg ctgcaaaaga
601 acctaagatc cccaatgctg atgaaataat cccgccaaac aaagcagcgg ttgccgcggc
661 ttttttttcc tcaatagctt tattctggct ctcaaaaacg gcctgctgaa tctgatagct
721 ttgcgccaat tgtttttggt ggtaatcctg caatagagtc accatcttgc cgaggagtgt
781 ttgaatttcc agcatcagct tacaaatata aaccttacca tcagtaagca gagatgaatc
841 aattgataaa gcagatgcgg caactgcact ggaagcgccg gtcgtactct ccgaagcgga
901 attaaccatc gttacttgag tattatcaat agtattcat

```

A

```

1  MNTIDNTQVTMVNSASESTTGASSAVAASALSIDSSLLTDGKVDICKLML
51  EIQKLLGKMVTLLQDYQQKQLAQSYQIQQAVFESQNKAIIEKKAAATAAL
101 VGGIISSALGILGSFAAMNNAAKGAGEIAEKASSASSKAAGAASEVANKA
151 LVKATESVADVAAEEASSAMQKAMATTTKAASRASGVADDVAKASDFAEDL
201 ADAAEKTSRINKLLNSVDKLTNTTAFVAVTSLAEGTKTLPTTISESVKST
251 HEVNEQRAKSLENFQQGNLELYKQDVRRTQDDITTRLRDITS AVRDLLEV
301 QNRMGQSGRLAG

```

B**FIGURE 17**

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```

1  ttaaattcgg ccactaacaa tacgactatt taccctgtgct gaatcggaca tcagttgaga
61 aacactttgt aaatagctcg cctgattttg taactcgctt gccgctttat ccagctcgag
121 cttegcactc tcacctaagc tttcactctg tcgagttaag ttttgcaccg cgaaagcagc
181 taactgagac aacatttgga gttgcgcagc ctcatatttc aacgcagttg tcccggcaga
241 acgaataaccg ttcgtaacgc cctcagccac tgtcgaaatt tttgcaaaaa cgtttttaac
301 cagcgcttct gccgccttct caacgacttt tacaacgctt gagccaattt tgtagcaac
361 attaccaaatt ttagataaca gtgaagacac cccgccaacg ccggctgtca gaatgcttgc
421 ggccatagat attccgcca aagacctgtgc tgcagctttt aatccctgag gggcattttc
481 cccattaca tcgactgccg tttgcagtgc cattgctgtt gcaccaatgg caacaacagc
541 ccagagtgtc gggttaaaaa cagcggcaac ggctgttaat gcgacgcca accaaccaaa
601 gacctggcca acaattttac ttttttgtga tttctcttcg gctttctgtt gttcttcgag
661 ctgtttttta tactcctgcg ttttattctc cagcgcttta gtttgcccat ccatataaat
721 ctcgtagag tttttcagac tcgagacttt ctgcgcggaa gtatccaggg ataacagagt
781 gaccatcatc atcatttgct gaggggtcaac ggtattcacc tgagagagat agggatagct
841 tgtgcgctgt ggctctgcct cctcagtgcg acttgtaacc tcaccactaa taccaccaa
901 caatttacta aggacatcct cagcagcaga gggcgctact aatgagtgc ctgccggcgg
961 cgtcggtaaa gggctacttt ctgtccagcc tgctgacgag ttcattggatt taaccagttg
1021 taaatccagc gataaaccg tttcagattg agtaatacca gaagtaccg aggcggtatt
1081 aacccagac gttacagaca gggtatcgtt atttacgtta agcat

```

A

```

1  MLNVNNDTLSVTSGVNTASGTSGITQSETGLSLDLQLVKSMNSSAGWTES
51  SPLPTPPAGHSLVTPSAAEDVLSKLFGGISGEVTSRTEEAEPQRTSYPYL
101 SQVNTVDPQQMMMMVTLLSLDTSQKVSSLKNSNEIYMDGQTKALENKTO
151 EYKKQLEEQQKAEKSKQSKIIVGQVFGWLGVALTAVAAVFNPALWAVVAI
201 GATAMALQTAVDVMGENAPQGLKTAQVFGGISMAASILTAGVGGVSSLL
251 SKFGNVANKIGSSVVKVVEKAAEALVKNVFAKISTVAEGVTNGIRSAGTT
301 ALNNEAAQLQMLSQLAAFAVQNLTRQSESLGESAKLELDKAASELQNQAS
351 YLQSVSQLMSDSARVNSRIVSGRI

```

B**FIGURE 18**

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```

1  atgaacattc aaccgaccat acaatctgga atcacctcac aaaacaatca acatcatcaa
61  acagaacaaa taccctctac acaaataccg caatccgaat tacctctagg atgccaagct
121 ggatttggtg ttaatatcc agatgatata cagcaacatg caccggaatg cggtgaaaca
181 acagctctac tgagcttgat aaaagataaa ggtctgctct cagggctaga cgaatatata
241 gctcctcacc ttgaagaagg atccatagga aaaaaaacat tggatatgtt tggtttattc
301 aatggttacc aaatggcatt agagatacct agttccgttt caggcatctc tggtaaatat
361 ggtgtccagc taaacattgt aaaaccagat attcatccta catcaggtaa ttatttttta
421 cagatatcc ctctgcatga tgaaatagg ttttaatttta aagaccttcc tggcccgtta
481 aaaaatgcat taagcaacag taatatatca accactgcag tgtcgactat tgcacgact
541 ggaacatcag ccactacttc gacggtaacc accgagccaa aagacccaat accatggttt
601 ggattaacag ctcaagtggg tcgtaatcat ggtgtagaac ttcctatagt caaaactgaa
661 aatggatgga agcttggttg agaaacacca cttactcctg atgggccgaa agcaaattac
721 acggaggagt gggttatcag accgggagaa gcagatttta aatatgggtg atctccatta
781 caggcaactc tagggctgga gtttggcgca catttcaagt gggatttaga taacccta
841 actaaatatg ccgttcttac caatgctgcc gcaaatgcgc ttggtgcttt agggggattt
901 gcagtatcca gatttgctag tacagatcca atgttaagtc ctcatatcgg tgcaatggtt
961 gggcaagcag cagggcattg catacagtat aataccctg gattaaagcc agacactatt
1021 ttatgggtgg ctggtgagc actgggggct gccgatttaa acaaggccga gtttgaagta
1081 gctagattca ctgactatcc tcgtatatgg tggcacgcaa gagaaggagc tattttcccc
1141 aataaagcag atattgaaca tgccacaggt gctgatatac gcgcaatgga agaaggatc
1201 cctggtggac agcggcatcc aaatccagag gatgtggtta tcgatatcga aagcaatggc
1261 ttaccacatc ataatccatc aaatcatgtt gatatctttg atataatcca agaaacaaga
1321 gtctaa

```

A

```

1  MNIQPTIQSGITSQNNQHHQTEQIPSTQIPQSELPLGCQAGFVVNIIPDDI
51  QQHAPECGETTALLSLIKDKGLLSGLDEYIAPHLEEGSIGKKTLDMFGLF
101 NVTQMALEIPSSVSGISGKYGVQLNIVKPDHPTSGNYFLQIFPLHDEIG
151 FNFKDLPGPLKNALSNSNISTTAVSTIASTGTSATTSTVTTEPKDPI PWF
201 GLTAQVVRNHGVELPIVKTENGWKLVGETPLTPDGPKANYTEEWVIRPGE
251 ADFKYGASPLQATLGLEFGAHFKWDLNPNNTKYAVLTNAAANALGALGGF
301 AVSRFASTDPMLSPHIGAMVGQAAGHAIQYNTPLKPDILWWAGATLGA
351 ADLNKAEFEVARFTDYPRIWWHAREGAI FPNKADIEHATGADIRAMEEGI
401 PVGQRHPNPEDVVIDIESNGLPHHNPSNHVDIFDIIQETRV

```

B**FIGURE 19**

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```

1 atgatacttg ttgccaaatt gttcattaca aaccagatag gagaatctct catgataaat
61 ggacttaata atgactccgc atcttttagtt ttagatgctg caatgaaagt taattctggg
121 tttaaaaaaa gctgggatga gatgtcatgc gctgaaaagt tatttaaagt acttagtttt
181 ggtttatgga atccaacgta cagtcgtagt gaaagacaat catttcaaga gttgttaacc
241 gtttttagagc ctgtatatcc acttcccaat gaattaggca gagtatctgc tcgtttttca
301 gatggttcat ccttaagaat ttccgtcact aacagcgaac ttggtgaagc cgagattcgc
361 acagcaaata atgaaaagat tactgtgctc ctggagtcaa acgaacaaaa taggttatta
421 caatctttac ccatcgatcg ccacatgcca tacattcagg ttcatcgtgc cttatctgag
481 atggacctga ctgatactac ctcaatgcgc aatctacttg gttttacgtc aaaactatca
541 acaaccttga ttcctcataa tgctcaaaca gatccgcttt ccgggcctac accattcagc
601 tctatcttta tggatacatg tgcaggactc ggcaatgcaa agctttcact caatggtggt
661 gatatacctg caaatgcaca aaaattgctt cgcgatgcac taggacttaa agacacacat
721 tcatcaccaa cccggaatgt tatagatcat ggtatttctc gccatgatgc agagcaaata
781 gcaagagaaa gcagcggcag tgataaacag aaagctgaag ttgtggaatt tttatgccat
841 ccagaagcag caacggccat atgctcggct ttctatcaat ctttcaatgt gccagcctta
901 acgttgacac atgaaaggat ctctaaagcc agtgaatata atgcggaaag atcattagat
961 acacctaacg cttgcattaa catcagtatc tctcaatcat cagatggaaa catttatggt
1021 accagccata ctgggggttct gataatggcg ccagaagacc gcccacacga gatgggcatg
1081 ttgacgaaca ggacttctta tgaagtgccg caaggtgtga aatgtataat cgatgaaatg
1141 gtaagtgcgc tacaaccaag gtatgccgca tctgaaacgt acttacaaaa cacttaa

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A

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1 MILVAKLFITNQIGESLMINGLNNDASLVLDAAAMKVNSGFKKSWDEMSC
51 AEKLFKVLSEFGLWNPTYRSERQSFQELLTVLEPVYPLPNELGRVSARFS
101 DGSSLRISVTNSELVEAEIRTANNEKITVLLNESNEQNRLQLSLPIDRHMP
151 YIQVHRALSEMDLTDTTSMRNLLGFTSKLSTTLIPHNAQTDPLSGPTPFS
201 SIFMDTCRGLGNAKLSLNGVDIPANAQKLLRDALGLKDTHTSSPTRNVIDH
251 GISRHDAEQIARESSGSDKQKAEVVEFLCHPEAATAICSAFYQSFNVPAL
301 TLTHERISKASEYNAERSLDTPNACINISISQSSDGNIIYVTSHTGVLIMA
351 PEDRPNEMGMLTNRTSYEVPQGVKCIIDEMVSALQPRYAASETYLQNT

```

B**FIGURE 20**

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```
1  atgattaatc ctgttactaa tactcagggc gtgtccccta taaatactaa atatgctgaa
61 catgtggtga aaaatatatta cccggaaatt aaacatgatt actttaatga atcacccaat
121 atatatgata agaagtatat atccggtata accagaggag tagctgaact aaaacaggaa
181 gaatttggtta acgagaaagc cagacgggtt tcttatatga agactatgta ttctgtatgt
241 ccagaagcgt ttgaacctat ttccagaaat gaagccagta caccggaagg aagctggcta
301 acagttatat ccggaaaacg cccaatgggg cagttttctg tagatagttt atacaatcct
361 gatttacatg cattatgtga gcttccggac atttggtgta agatcttccc taaagaaaat
421 aatgattttt tatacatagt tgttgtgtac agaaatgaca gccctctagg agaacaacgg
481 gcaaatagat ttatagaatt atataatata aaaagagata tcatgcagga attaaattat
541 gagttaccag agttaaaggc agtaaaatct gaaatgatta tcgcacgtga aatgggagaa
601 atcttttagct acatgcctgg ggaaatagac agttatatga aatacataaa taataaactt
661 tctaaaattg agtag
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A

```
1  MINPVTNTQGVSPINTKYAEHVVKNIYPEIKHDYFNESPNIYDKKYISGI
51  TRGVAELKQEEFVNEKARRFSYMKTMYSVCPEAFEPISRNEASTPEGSWL
101 TVISGKRPMGQFSVDSLNPDLHALCELPDICCKIFPKENNDFLYIVVVY
151 RNDSPLEQRANRFIELYNIKRDIMQELNYELPELKAVKSEMI IAREMGE
201 IFSYMPGEIDSYMKYINNKLKSKIE
```

B**FIGURE 21**

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1 atgttatcgc catattctgt aaatttgga tgttcattga attctttaac cagaaacctg
61 acttcgcctg ataatcgtgt ttatcctct gtaagggatg ctgccgttca ttctgataat
121 ggggcgcaag taaagggttg caacagaaca tatcgtgttg ttgccaccga taataagttt
181 tgcgttacaa gagaaagtca tagtggttgt ttactaatc tgttgcacag gctgggatgg
241 cctaaggggg agattagcag gaaaattgag gtcattgctga atgcatcacc agtgagcgc
301 gctatggaaa gaggcattgt tcattcgaac agacctgatt tacctcctgt tgattatgca
361 ccgccagagt taccgagtgt ggactataac aggttgctcag tacctggtaa tgttattggc
421 aaagggggga acgctgtagt atatgaagat gctgaggatg caacaaaagt cctgaagatg
481 ttactacat ctcaaagcaa tgaagaggtg acaagcgaag ttcgttgctt caaccaatat
541 tatggtgccg ggagtgcaga aaaaatatat ggcaataatg gtgatattat tggattaga
601 atggataaaa taaatggaga atcgctttta aatatttcgt ccttgccagc acaggctgag
661 catgctatctt acgatatgtt tgatagactg gagcaaaaag gaattctttt tgcgataca
721 acagagacaa atgtcttata tgaccgcgcg aagaatgagt ttaatccaat agatatatca
781 tcttataatg tttccgaccg ttcattggagt gaaagtcaaa taatgcaatc ttatcatggc
841 ggaaagcaag atcttattag tgtggtatta agtaaaattt ag

A

1 MLSPYSVNLGCSWNSLTRNLTPDNRLSSVRDAAVHSDNGAQVKVGNRT
51 YRVVATDNKFCVTRESHSGCFTNLLHRLGWPKGEISRKIEVMLNASPVSA
101 AMERGIVHSNRPDLPVDYAPPELPSVDYNRLSVPGNVIGKGGNAVVED
151 AEDATKVLKMF TTSQSNEEVTSEVRCFNQYYGAGSAEKIYGNNGDIIGIR
201 MDKINGESLLN ISSLPAQAEHAIYDMFDRLEQKGILFVDTTETNVLYDRA
251 KNEFNPIDISSYNVSDRSWSESQIMQSYHGGKQDLISVVLSKI

B**FIGURE 22**

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1 atgttatcgc catattctgt aaatttggga tgttcatgga attctttaac cagaaacctg
61 acttcgcctg ataatcgtgt ttatccctct gtaagggatg ctgccgttca ttctgataat
121 ggggcgcaag taaagggttg caacagaaca tatcgtgttg ttgccaccga taataagttt
181 tgcgttacaa gagaaagtca tagtggttgt ttactaatc tgttgcacag gctgggatgg
241 cctaaggggg agattagcag gaaaattgag gtcattgctga atgcatcacc agtgagcgct
301 gctatggaaa gaggcattgt tcattcgaac agacctgatt tacctcctgt tgattatgca
361 ccgccagagt taccgagtgt ggactataac aggttgctcag tacctggtaa tgttattggc
421 aaagggggga acgctgtagt atatgaagat gctgaggatg caacaaaagt cctgaagatg
481 ttactacat ctcaaagcaa tgaagaggtg acaagcgaag ttcgttgctt caaccaatat
541 tatggtgccg ggagtgcaga aaaaatatat ggcaataatg gtgatattat tggattaga
601 atggataaaa taaatggaga atcgctttta aatatttcgt ccttgccagc acaggctgag
661 catgctattt acgatatgtt tgatagactg gagcaaaaag gaattctttt tgcgataca
721 acagagacaa atgtcttata tgaccgcgcg aagaatgagt ttaatccaat agatatatca
781 tcttataatg tttccgaccg ttcattggagt gaaagtcaaa taatgcaatc ttatcatggc
841 ggaaagcaag atcttattag tgtggtatta agtaaaattt ag

A

1 MLSPYSVNLGCSWNSLTRNLTPDNRVLSSVRDAAVHSDNGAQVKVGNRT
51 YRVVATDNKFCVTRESHSGCFTNLLHRLGWPKGEISRKIEVMLNASPVSA
101 AMERGIVHSNRPDLPVDYAPPELPSVDYNRLSVPGNVIGKGGNAVVED
151 AEDATKVLKMFTTSQSNEEVTSEVRCFNQYYGAGSAEKIYGNNNGDIIGIR
201 MDKINGESLLNISSLPAQAEHAIYDMFDRLEQKGILFVDTTETNVLYDRAK
251 NEFNPIDISSYNVSDRSWSESQIMQSYHGGKQDLISVVLSKI

B**FIGURE 23**

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1 ttaccctttc ttcgattgct cataggcagc taaatgatct tttaatgcct gtgcaagggg
61 cggtagtcca ctaggccctg tcggtggcgg tggtagcctga cgggcgggct taaaacctaa
121 agcctcagga cctttcgatt tttcataggc agccaagtgc tcttttaatg cctgtgcaat
181 gggcggtaaa ggtcgggatg ccccgatgc ctgtccactt gtcggtggcg gcggtgcctg
241 acgggcgggc ttaaaaccta aagcctcagg acctttcgat ttttcatagg cagccaagtg
301 ctctttttaat gcctgtgcaa tgggcggtaa aggtcgggat gccccggatg cctgtccact
361 tgtcgggtggc ggcggtgcct gacgggcggg cttaaaacct aaagcctcag gacctttcga
421 tttttcatag gcagccaagt gctcttttaa tgcctgtgca atgggcggta aaggtcggga
481 tgccccggat gcctgtccac ttgtcgggtg cggcgggtgcc ggacgagagg gagtaaata
541 agtcacctgg ctgctcacat taaaaatcgt tctcgatta acattcgacg agcctggaga
601 aaaaggggaa tgaactttca ccggagtaag acgcacggcc tgaggggcta cagaaaatcc
661 agttcccccc gcagagctca ctcgacttgc gataacctaca agctgccgcc ctagtgtaga
721 agcagcggtta ctaattccat taagcat

A

1 MLNGISNAASTLGRQLVGIASRVSSAGGTGFSVAPQAVRLTPVKVHSPFS
51 PGSSNVNARTIFNVSSQVTSFTPSRPAPPPPTSGQASGASRPLPPIAQAL
101 KEHLAAYEKSKEALGFKPARQAPPPPTSGQASGASRPLPPIAQALKEH
151 LAAYEKSKEALGFKPARQAPPPPTSGQASGASRPLPPIAQALKEHLAA
201 YEKSKEALGFKPARQAPPPPTGPSGLPPLAQALKDHLAAYEQSKKG

B**FIGURE 24**

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1  atgaaattcc cttcaatatt taacaaaata aaaccacaat ccatacagca acatccagaa
61  aaaaatcaac ttaactggat gctcgaatta aataaatgga aagaagaacg tatacttaca
121 ggtgaaatcc atcgtccgga atgtcgaaac gaagccgcta aaaggataaa ctgtgctttt
181 ttgtcgaaac agaatgacat tgatttatca ggacttaatt tatctactca accaccaggg
241 ctgcaaaaact tcacctctat caatcttgat aataaccaac tcacacattt tgatgcaacc
301 aactacgata gactcgtaaa acttagtctg aatagtaaca ctcttgagtc aataaatatt
361 catcaaggca gaaatgtaag cattacacat atatctatga ataataattg tctcagaaat
421 attgatatag ataggctttc atcaattact tatttttagtg cggcacataa taaactagag
481 tttgtgcaat tagaatcttg cgaatggctg caatacctga atctcagcca taatcaatta
541 actgatattg ttacaggaaa taaagaagaa ctcttactgc tggatctatc ccataataaa
601 ctagcaagtt tacacaatgc cttattttccc aacttaaata cgttacttat caacaacaac
661 ttgcttttctg aaattaaaaat gttttatagc aacttctgca aagttcagac attaaacgct
721 gctaacaatc agttggaaaa aataaacctt catttcctga cttatctttc atctatcaaa
781 agtttaaggc tggacaataa taaaataact cgcattgata ctgagaacac atccgatatt
841 agaagtttat tccccataat aaagaagagc gaaagcttaa atttttttaa tatttctggc
901 gagaacaatt gccctactat ccagctcatg ttattttaatt tgttttcccc agcacttaag
961 cttaatactg gcctggcaat tctttcgctt ggtgcatttg aagatcactc tgacggatta
1021 gatgtggata acgaattggt tcactatact attaataaag catatacccc atataatata
1081 catacttata aaacagaaga agttgtaaac cagaggaata taaaaattaa aaatatgacc
1141 ttagatgaaa taaacaatac ttattgtaat aacgattatt acaatgaggc aataagagag
1201 gaaccgatag actttctgga cagatcggtt tcctccagct catggccttt ttatcactaa

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A

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1  MKFPSIFNKKIKPQSIQQHPEKNQLNWMLELNKWKEERILTGEIHRPECRN
51  EAAKRINCAFLSKQNDIDL SGLNLSTQPPGLQNFTSINLDNNQLTHFDAT
101 NYDRLVKLSLNSNTLESINI HQGRNV SITHISMNNCLRNIDIDRLSSIT
151 YFSAAHNKLEFVQLESC EWLQYLNLSHNQLT DIVTGNKEELLLDL SHNK
201 LASLHNALFPNLNTLLINNNLLSEIKMFYSNFCKVQTLNAANNQLEKINL
251 HFLTYLSSIKSLRLDNNKITRIDTENTS DIRSLFP IIKKSES LNFLN ISG
301 ENNCPTIQMLFNLFS PALKLN TGLAILSPGAFEDHSDGLDVDNELFHYT
351 INKAYTPYNIHTYKTEE VVNQRNI KIKNMTLDEINNTYCNNDYYNEAIRE
401 EPIDFLDRSFSSSSWPFYH

```

B**FIGURE 25**

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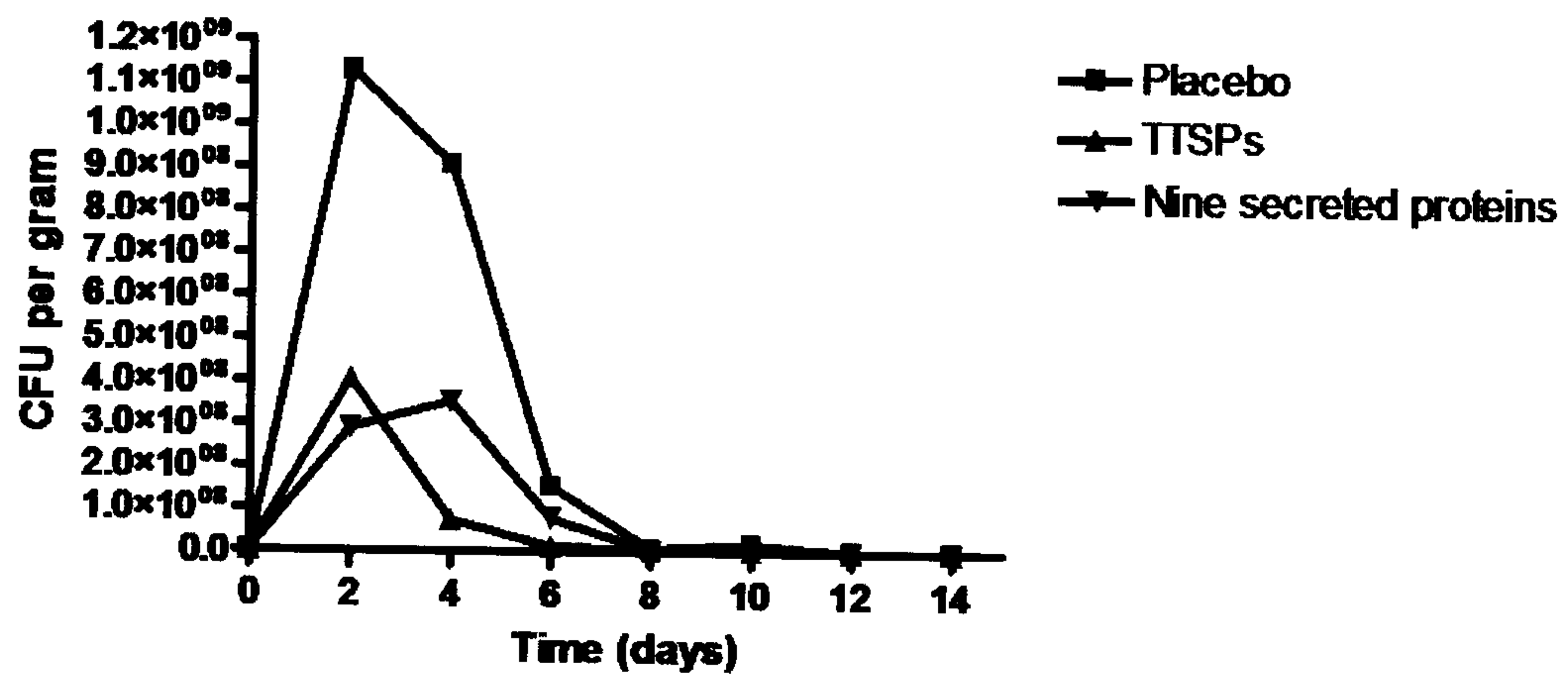


FIGURE 26