Immuno-Based Botulinum Toxin Serotype A Activity Assays

[01] This patent application claims priority pursuant to 35 U.S.C. § 119(e) to U. S. Provisional Patent Application Serial No. 61/036,723 filed March 14, 2008, which is hereby incorporated by reference in its entirety.

[02] The ability of Clostridial toxins, such as, *e.g.*, Botulinum neurotoxins (BoNTs), BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F and BoNT/G, and Tetanus neurotoxin (TeNT), to inhibit neuronal transmission are being exploited in a wide variety of therapeutic and cosmetic applications, see e.g., William J. Lipham, Cosmetic and Clinical Applications of Botulinum Toxin (Slack, Inc., 2004). Clostridial toxins commercially available as pharmaceutical compositions include, BoNT/A preparations, such as, e.g., BOTOX® (Allergan, Inc., Irvine, CA), DYSPORT®/RELOXIN®, (Ipsen Ltd., Slough, England), PURTOX® (Mentor Corp., Santa Barbara, CA), XEOMIN® (Merz Pharmaceuticals, GmbH., Frankfurt, Germany), NEURONOX® (Medy-Tox, Inc., Ochang-myeon, South Korea), BTX-A (Biogen-tech Ltd., University, Yantai, Shandong, China); and BoNT/B preparations, such as, e.g., MYOBLOC®/NEUROBLOC® (Solstice Neurosciences, Inc., South San Francisco, CA). As an example, BOTOX® is currently approved in the U.S. for the treatment of cervical dystonia in adults to decrease the severity of abnormal head position and neck pain associated with cervical dystonia; for the treatment of severe primary axillary hyperhidrosis that is inadequately managed with topical agents; and for the treatment of strabismus and blepharospasm associated with dystonia, including benign essential blepharospasm or VII nerve disorders in patients 12 years of age and above.

[03] At present the mouse LD_{50} bioassay, a lethality test, remains the "gold standard" used by all pharmaceutical manufacturers to express the potency of their preparations. S. S. Arnon et al., JAMA 285: 1059-1070 (2001). In fact, the units on the pharmaceutical preparations' labels are mouse LD_{50} units and the number of animals needed to produce statistically useful LD_{50} data is large. The advantage of the mouse LD_{50} bioassay is that it measures all the steps necessary for botulinum toxin uptake (*e.g.*, toxin binding to a cell surface receptor, internalization of the toxin-receptor complex, light chain translocation into the cytoplasm, light chain cleavage of substrate), instead of merely determining the activity for only part of this intoxication process, such as, *e.g.*, in vitro assays that only measure light chain enzymatic activity. Unfortunately, the mouse LD_{50} bioassay suffers from many drawbacks including high operational cost due to the large numbers of laboratory animals required, a lack of specificity since all BoNT serotypes will cause the same measurable end-point, and the potential for inaccuracy unless large animal groups are used. In addition, animal rights groups have exerted pressure on regulatory agencies in the U.S. (FDA/NICEATM/ICCVAM) and Europe (MHRA and EDQM), and on pharmaceutical companies manufacturing botulinum neurotoxin products to reduce animal testing and more importantly replace the mouse LD_{50} bioassay for product release. The regulatory agencies are engaging pharmaceutical companies to apply the three "Rs" principle to the potency testing of botulinum neurotoxins: Reduce, Refine, Replace. D. Straughan, *Progress in Applying the Three Rs to the Potency*

Testing of Botulinum Toxin Type A, Altern. Lab. Anim. 34(3): 305-313 (2006). In recent years, several steps have been already taken to reduce and refine the mouse LD_{50} bioassay in order to standardize the protocol and produce more consistent data using fewer animals per assay.

[04] Thus, a simple, reliable, validated and governmental agency acceptable botulinum toxin activity assay that can evaluate the integrity of all the steps necessary in botulinum toxin uptake would be of significant value because such a non-animal based assay would alleviate the need for animal testing and all the disadvantages, costs and ethical concerns associated with this type of animal-based assay. The present specification provides novel compositions, cells, and methods for assaying the activity of a botulinum toxin A useful for various industries, such as, *e.g.*, the pharmaceutical and food industries, and provides related advantages as well. Such compositions, cells, and methods do not use live animals or tissues taken from live animals, but can evaluate all the steps necessary for neurotoxin action.

DETAILED DESCRIPTION OF THE DRAWINGS

[05] **FIG. 1** shows a schematic of the current paradigm of neurotransmitter release and Clostridial toxin intoxication in a central and peripheral neuron. FIG. 1A shows a schematic for the neurotransmitter release mechanism of a central and peripheral neuron. The release process can be described as comprising two steps: 1) vesicle docking, where the vesicle-bound SNARE protein of a vesicle containing neurotransmitter molecules associates with the membrane-bound SNARE proteins located at the plasma membrane; and 2) neurotransmitter release, where the vesicle fuses with the plasma membrane and the neurotransmitter molecules are exocytosed. FIG. 1B shows a schematic of the intoxication mechanism for tetanus and botulinum toxin activity in a central and peripheral neuron. This intoxication process can be described as comprising four steps: 1) receptor binding, where Clostridial toxin binds to a Clostridial receptor complex and initiates the intoxication process; 2) complex internalization, where after toxin binding, a vesicle containing a toxin/receptor system complex is endocytosed into the cell; 3) light chain translocation, where multiple events are thought to occur, including changes in the internal pH of the vesicle, formation of a channel pore comprising the H_N domain of Clostridial toxin heavy chain, separation of the Clostridial toxin light chain from the heavy chain, and release of the light chain and 4) enzymatic target modification, where the light chain of Clostridial toxin proteolytically cleaves its target SNARE substrates, such as, *e.g.*, SNAP-25, VAMP or Syntaxin, thereby preventing vesicle docking and neurotransmitter release.

[06] **FIG. 2** shows a comparison of BoNT/A uptake in four cell lines by Western blot analysis. FIG. 2A shows a graph of SNAP-25 cleavage product detected based ~~on~~ amount of BoNT/A used to treat the cell line. The data were analyzed in SigmaPlot using a 4 parameter logistic model and EC_{50} values were obtained for each cell line. Ranking of SNAP-25 cleavage product signals detected was: SiMa>> Neuro-2a>LA1-55n> PC12. FIG. 2B shows the signal-to-noise ratios of the raw signals at 300 pM vs. 0 pM and

1.2 pM vs. 0 pM were calculated for the assay. SiMa cells generated the highest signal-to-noise ratios and the lowest EC_{50} values.

[07]FIG. 3 shows optimization of cell differentiation media for established cell lines useful in an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton.

[08]FIG. 4 shows optimization of cell differentiation time for cells comprising an established cell line useful in an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton.

[09]FIG. 5 shows optimization of BoNT/A treatment of cells comprising an established cell line useful in an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton. The results indicate an EC_{50} of less than 2 pM was achieved with any of the BoNT/A treatments tested.

[010] FIG. 6 shows the sensitivity of an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton. The results indicated that uptake of BoNT/A by the cells took less than one minute before producing significant amounts of SNAP-25 cleavage product over background.

[011] FIG. 7 shows the specificity of an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton. The results indicate that the immuno-based methods of detecting BoNT/A activity disclosed in the present specification can measure all the steps involved in BoNT/A intoxication.

[012] FIG. 8 shows a dose response curve of differentiated SiMa cells treated with a BoNT/A complex using an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton.

[013] FIG. 9 shows the results of an immuno-based BoNT/A activity assay for a formulated BoNT/A pharmaceutical product using an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton.

[014] FIG. 10 show the detection of neutralizing α-BoNT/A antibodies in human serum using an immuno-based method of detecting BoNT/A activity disclosed in the present specificaiton.

DETAILED DESCRIPTION

[015] The present specification provides novel assays for determining the presence or absence of an active BoNT/A in a sample and for determining the activity/potency of a BoNT/A preparation. The novel cell-based assays disclosed in the present specification rely on cells, reagents and detection methods that enable the assay to detect picomolar quantities of BoNT/A in a sample. The cell-based assays disclosed in the present specification reduce the need for animal toxicity studies, yet serve to analyze multiple functions BoNT/A, namely, binding and cellular uptake of toxin, translocation into the cell cytosol,

and protease activity. As discussed further below, the novel methods and compositions can be used to analyze crude and bulk samples as well as highly purified di-chain toxins and formulated toxin products and further are amenable to automated high throughput assay formats.

[016] Thus, one aspect disclosed in the present specification provides compositions for producing α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. Compositions can comprise an adjuvant and a composition including a SNAP-25 antigen, a carrier linked to a SNAP-25 antigen, or a carrier linked to a flexible spacer linked to a SNAP-25 antigen, where the flexible linker intervenes between the SNAP-25 antigen and the carrier. It is envisioned that any and all SNAP-25 antigens that triggers an immune response that produce a α-SNAP-25 antibody that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be useful as a SNAP-25 antigen, including, without limitation, a SNAP-25 antigen derived from a naturally occurring SNAP-25, a SNAP-25 antigen derived from a non-naturally occurring SNAP-25, and a SNAP-25 antigen comprising an immunoreactive fragment of the SNAP-25, the SNAP-25 from a naturally occurring SNAP-25 or a non-naturally occurring SNAP-25. SNAP-25 antigens useful for producing α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product include, without limitation, SNAP-25 antigens comprising a SNAP-25 peptide having a carboxylated C-terminal glutamine linked to a carrier peptide, including, without limitation SEQ ID NO: 38. Other compositions useful for making α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product include, without limitation, a composition comprising a carrier linked to a flexible linker linked to a SNAP-25 antigen a carboxylated C-terminal glutamine, wherein the flexible linker intervenes between the SNAP-25 antigen and the carrier. It is envisioned that any and all adjuvants can be useful in such a composition, including, without limitation, polyethylene glycol (PEG), monomethoxypolyethylene glycol (mPEG), polyvinyl alcohol (PVA), complete and incomplete Freund's adjuvant.

[017] Another aspect disclosed in the present specification provides methods of producing an α-SNAP-25 antibody that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. Aspects of this method comprise the steps of (a) administering to an animal a composition disclosed in the present specification; (b) collecting from the animal a sample containing an α-SNAP-25 antibody or α-SNAP-25 antibody-producing cell; and (c) isolating the α-SNAP-25 antibody from the sample. The methods disclosed are useful for making either α-SNAP-25 monoclonal antibodies that can bind an epitope comprising a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product or α-SNAP-25 polyclonal antibodies that can bind an epitope comprising a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product.

[018] Still another aspect disclosed in the present specification provides α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. Such α-SNAP-25 antibodies include both naturally-occurring and non-naturally-occurring antibodies, as well as, monoclonal α-SNAP-25 antibodies or polyclonal α-SNAP-25 antibodies. Monoclonal α-SNAP-25 antibodies useful as α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, include, without limitation, the monoclonal α-SNAP-25 antibodies produced from hybridoma cell lines 1D3B8, 2C9B10, 2E2A6, 3C1A5 and 3C3E2.

[019] Yet another aspect disclosed in the present specification provides methods of detecting BoNT/A activity. Aspects of this method comprise the steps of (a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line is susceptible to BoNT/A intoxication; (b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; (c) contacting the SNAP-25 component with an α-SNAP-25 antibody that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and (d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity. The α-SNAP-25 antibody of step c can optionally be linked to a solid phase support.

[020] Yet another aspect disclosed in the present specification provides methods of detecting BoNT/A activity. Aspects of this method comprise the steps of (a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line can uptake a BoNT/A; (b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; (c) contacting the SNAP-25 component with an α-SNAP-25 antibody that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and (d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity. The α-SNAP-25 antibody of step c can optionally be linked to a solid phase support.

[021] A further aspect disclosed in the present specification provides methods of determining BoNT/A immunoresistance in a mammal. Aspects of this method comprise the steps of (a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; (b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line is susceptible to BoNT/A intoxication; (c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; (d) contacting the SNAP-25 component with an α-SNAP-25 antibody

that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; (e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; (f) repeating steps a-e with a negative control sample instead of a test sample; and (g) comparing the amount of antibody-antigen complex detected in step (e) to the amount of antibody-antigen complex detected in step (f), wherein detection of a lower amount of antibody-antigen complex detected in step (e) relative to the amount of antibody-antigen complex detected in step (f) is indicative of the presence of α-BoNT/A neutralizing antibodies. The α-SNAP-25 antibody of step d can optionally be linked to a solid phase support. The control sample in step f can also include a positive control sample, in addition to the negative control sample.

[022] Clostridia toxins produced by *Clostridium botulinum*, *Clostridium tetani*, *Clostridium baratii* and *Clostridium butyricum* are the most widely used in therapeutic and cosmetic treatments of humans and other mammals. Strains of *C. botulinum* produce seven antigenically-distinct serotypes of botulinum toxins (BoNTs), which have been identified by investigating botulism outbreaks in man (BoNT/A, BoNT/B, BoNT/E and BoNT/F), animals (BoNT/C1 and BoNT/D), or isolated from soil (BoNT/G). While all seven botulinum toxin serotypes have similar structure and biological properties, each also displays heterogeneous characteristics, such as, *e.g.*, different pharmacological properties. In contrast, tetanus toxin (TeNT) is produced by a uniform group of *C. tetani*. Two other species of Clostridia, *C. baratii* and *C. butyricum*, also produce toxins similar to BoNT/F and BoNT/E, respectively.

[023] Clostridial toxins are each translated as a single chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease, such as, e.g., an endogenous Clostridial toxin protease or a naturally-occurring protease produced in the environment. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single disulfide bond and noncovalent interactions. Each mature di-chain molecule comprises three functionally distinct domains: 1) an enzymatic domain located in the LC that includes a metalloprotease region containing a zinc-dependent endopeptidase activity which specifically targets core components of the neurotransmitter release apparatus; 2) a translocation domain contained within the amino-terminal half of the HC (H_N) that facilitates release of the LC from intracellular vesicles into the cytoplasm of the target cell; and 3) a binding domain found within the carboxyl-terminal half of the HC (H_C) that determines the binding activity and binding specificity of the toxin to the receptor complex located at the surface of the target cell.

[024] The binding, translocation and enzymatic activity of these three functional domains are all necessary for toxicity. While all details of this process are not yet precisely known, the overall cellular intoxication mechanism whereby Clostridial toxins enter a neuron and inhibit neurotransmitter release is similar, regardless of serotype or subtype. Although the applicants have no wish to be limited by the

following description, the intoxication mechanism can be described as comprising at least four steps: 1) receptor binding, 2) complex internalization, 3) light chain translocation, and 4) enzymatic target modification (FIG. 1). The process is initiated when the HC domain of a Clostridial toxin binds to a toxin-specific receptor system located on the plasma membrane surface of a target cell. The binding specificity of a receptor complex is thought to be achieved, in part, by specific combinations of gangliosides and protein receptors that appear to distinctly comprise each Clostridial toxin receptor complex. Once bound, the toxin/receptor complexes are internalized by endocytosis and the internalized vesicles are sorted to specific intracellular routes. The translocation step appears to be triggered by the acidification of the vesicle compartment. This process seems to initiate important pH-dependent structural rearrangements that increase hydrophobicity, promote pore formation, and facilitate separation of the heavy and light chains of the toxin. Once separated, the light chain endopeptidase of the toxin is released from the intracellular vesicle into the cytosol where it appears to specifically target core components of the neurotransmitter release apparatus. These core proteins, vesicle-associated membrane protein (VAMP)/synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25) and Syntaxin, are necessary for synaptic vesicle docking and fusion at the nerve terminal and constitute members of the soluble N-ethylmaleimide-sensitive factor-attachment protein-receptor (SNARE) family. BoNT/A and BoNT/E cleave SNAP-25 in the carboxyl terminal region, releasing a nine or twenty six amino acid fragment, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxyl terminus releasing an eight amino acid fragment. The botulinum serotypes BoNT/B, BoNT/D, BoNT/F and BoNT/G, and tetanus toxin, act on the conserved central portion of VAMP, and release the amino terminal portion of VAMP into the cytosol. BoNT/C1 cleaves syntaxin at a single site near the cytosolic membrane surface. The selective proteolysis of synaptic SNAREs accounts for the block of neurotransmitter release caused by Clostridial toxins in vivo. The SNARE protein targets of Clostridial toxins are common to exocytosis in a variety of non-neuronal types; in these cells, as in neurons, light chain peptidase activity inhibits exocytosis, see, e.g., Yann Humeau et al., *How Botulinum and Tetanus Neurotoxins Block Neurotransmitter Release*, 82(5) Biochimie. 427-446 (2000); Kathryn Turton et al., *Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic Utility*, 27(11) Trends Biochem. Sci. 552-558. (2002); Giovanna Lalli et al., *The Journey of Tetanus and Botulinum Neurotoxins in Neurons*, 11(9) Trends Microbiol. 431-437, (2003).

[025] Aspects of the present disclosure comprise, in part, a composition for producing α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. Other aspects of the present disclosure comprise, in part, an immune response inducing composition for producing α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. As used herein, the term "immune response inducing composition" refers to a composition comprising a SNAP-25 antigen which, when administered to an animal, stimulates an immune response against the SNAP-25 antigen, thereby producing α-SNAP-25 antibodies that can bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A

cleavage site scissile bond from a SNAP-25 cleavage product. The term "immune response" refers to any response by the immune system of an animal to an immune response inducing composition. Exemplary immune responses include, but are not limited to, cellular as well as local and systemic humoral immunity, such as, *e.g.*, CTL responses, including antigen-specific induction of CD8+ CTLs, helper T-cell responses, including T-cell proliferative responses and cytokine release, and B-cell responses including, *e.g.*, an antibody producing response. The term "inducing an immune response" refers to administration of an immune response inducing composition or a polynucleotide encoding the immune response inducing composition, where an immune response is affected, *i.e.*, stimulated, initiated or induced.

[026] A composition comprises a SNAP-25 antigen. As used herein, the term "antigen" refers to a molecule that elicits an immune response and includes, without limitation, peptides, polysaccharides and conjugates of lipids, such as, *e.g.*, lipoproteins and glycolipids. As used herein, the term "SNAP-25 antigen" refers to any antigen which has a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond that can elicit an immune response. A SNAP-25 antigen used in an immune response inducing composition must be large enough to be substantially unique in sequence, thus reducing the possibility of producing antibodies that are cross reactive against antigens other than SNAP-25. In addition, a SNAP-25 antigen used in an immune response inducing composition must be small enough to only trigger an immune response substantially against a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, thus increasing the possibility of producing α-SNAP-25 antibodies that can distinguish a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. Furthermore, it is also very desirable to generate α-SNAP-25 antibodies of a single amino acid sequence in a good yield that are reproducibly selective and which bind with acceptable avidity in order to permit the design of a highly sensitive assay.

[027] The sequence surrounding a BoNT/A cleavage site present in SNAP-25 is denoted as P_5–P_4–P_3–P_2–P_1–P_1'–P_2'–P_3'–P_4'–P_5', with P_1–P_1' representing the scissile bond. Upon cleavage by BoNT/A, the resulting cleavage products produced comprise a fragment including the P_5–P_4–P_3–P_2–P_1 sequence and a fragment including the P_1'–P_2'–P_3'–P_4'–P_5'. Thus, as used herein, the term "SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond" refers to any SNAP-25 having the P_1 residue as its carboxyl-terminal amino acid. For example, Q_{197}–R_{198} of human SNAP-25 (SEQ ID NO: 5) represents the P_1–P_1' scissile bond for the BoNT/A cleavage site. As such, "SNAP-25 having a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond" would be any SNAP-25 cleavage product having a glutamine at its carboxyl-terminal amino acid where the glutamine represents Q_{197} of the scissile bond. As another example, K_{204}–H_{205} of *Torpedo marmorata* SNAP-25 (SEQ ID NO: 16) represents the P_1–P_1' scissile bond for the BoNT/A cleavage site. As such, "SNAP-25 having a carboxyl-terminus lysine of the BoNT/A cleavage site scissile bond" would be any SNAP-25 cleavage

product having a lysine at its carboxyl-terminal amino acid where the lysine represents K_{204} of the scissile bond.

[028] The SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from the BoNT/A cleavage site can be modified to enhance the immunogenicity of a SNAP-25 antigen, a hapten, or any other antigenic compound that is immunogenic, non-immunogenic, or weakly immunogenic when not associated with the modification. In an aspect of this embodiment, the carboxyl-terminal P_1 residue from the scissile bond of a SNAP-25 antigen can be carboxylated. Carboxylation increases the desired immunogenic properties of a SNAP-25 antigen in two respects. First, because charged amino acids enhance immunogenicity, adding a COO^- group to the carboxyl-terminal residue will increase the overall immunogenicity of a SNAP-25 antigen. Second, because the P_1 residue of the BoNT/A cleavage site scissile bond is in a charged state upon cleavage, adding a COO^- group to the carboxyl-terminal residue will better mimic the actual antigen that the α-SNAP-25 antibodies disclosed in the present specification are designed to bind.

[029] In an aspect of this embodiment, the amino-terminal residue from a SNAP-25 antigen can be modified by the addition of an amino acid adapted to attach the SNAP-25 antigen to a carrier protein, such as, *e.g.*, a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI), or a multiple attachment peptide (MAP). For example, a cysteine residue can be placed at the amino-terminus in order to conjugate the carrier protein KLH.

[030] Thus, an embodiment, a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can be, *e.g.*, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, or at least 30 amino acids in length. In another embodiment, a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can be, *e.g.*, at most 5, at most 6, at most 7, at most 8, at most 9, at most 10, at most 11, at most 12, at most 13, at most 14, at most 15, at most 16, at most 17, at most 18, at most 19, at most 20, at most 25, or at most 30 amino acids in length. In still another embodiment, a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can be, *e.g.*, between 7-12 amino acids, between 10-15 amino acids, or between 13-18 amino acids.

[031] In another embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 32. In aspects of this embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 147, or SEQ ID NO: 148. In a further embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 38.

[032] In yet another embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 39. In aspects of this embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44. In a further embodiment, the SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprises SEQ ID NO: 45.

[033] It is envisioned that any and all SNAP-25 antigens that triggers an immune response that produces α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be useful as a SNAP-25 antigen. Thus, amino acid sequence variants comprising SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 147, or SEQ ID NO: 148 can be useful as a SNAP-25 antigen to trigger an immune response that produces α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. Thus, in an embodiment, a SNAP-25 antigen can substitute at least 1, at least 2, at least 3, at least 4, or at least 5 amino acid substitutions, deletions or additions to the SNAP-25 antigens comprising SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 147, or SEQ ID NO: 148. In still another embodiment, a SNAP-25 antigen can have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity to the SNAP-25 antigens comprising SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 147, or SEQ ID NO: 148.

[034] It is envisioned that one or more carriers may be linked to a SNAP-25 antigen in order to enhance the immunogenicity of a SNAP-25 antigen that is immunogenic, non-immunogenic, or weakly immunogenic when not associated with the carrier. Non-limiting examples, include, *e.g.*, a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI), or a multiple attachment peptide (MAP). As is well known in the art, a non-antigenic or weakly antigenic antigen can be made antigenic by coupling the antigen to a carrier. Various other carrier and methods for coupling an antigen to a carrier are well known in the art. See, *e.g.*, Harlow and Lane, supra, 1998a; Harlow and Lane, supra, 1998b; and David W. Waggoner, Jr. et al., *Immunogenicity-enhancing carriers and compositions thereof and methods of using the same*, U.S. Patent Publication No. 20040057958 (Mar. 25, 2004). An epitope can also be generated by expressing the epitope as a fusion protein. Methods for expressing polypeptide fusions are well known to those skilled in the art as described, for example, in Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999). As the carboxyl-terminal end of the SNAP-25

antigen must be the P_1 residue of the BoNT/A cleavage site scissile bond, a carrier must be linked to the amino end of the SNAP-25 antigen.

[035] It is envisioned that one or more flexible spacers may be linked to a SNAP-25 antigen in order to enhance the immunogenicity of a SNAP-25 antigen that is immunogenic, non-immunogenic, or weakly immunogenic when not associated with the flexible linkers. A flexible spacer increases the overall peptide length of the SNAP-25 antigen and provides flexibility, thereby facilitating the proper presentation of the SNAP-25 antigen to the immune cells. As a non-limiting example, a composition can comprise a SNAP-25 antigen linked to one or more flexible spacers in tandem to better present SNAP-25 antigen to immune cells, thereby facilitating the immune response.

[036] A flexible space comprising a peptide is at least one amino acid in length and comprises non-charged amino acids with small side-chain R groups, such as, *e.g.*, glycine, alanine, valine, leucine or serine. Thus, in an embodiment a flexible spacer can be, *e.g.*, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 amino acids in length. In another embodiment, a flexible spacer can be, *e.g.*, at least 1, at most 2, at most 3, at most 4, at most 5, at most 6, at most 7, at most 8, at most 9, or at most 10 amino acids in length. In still another embodiment, a flexible spacer can be, *e.g.*, between 1-3 amino acids, between 2-4 amino acids, between 3-5 amino acids, between 4-6 amino acids, or between 5-7 amino acids. Non-limiting examples of a flexible spacer include, *e.g.*, a G-spacers such as GGG, GGGG (SEQ ID NO: 55), and GGGGS (SEQ ID NO: 56) or an A-spacers such as AAA, AAAA (SEQ ID NO: 57) and AAAAV (SEQ ID NO: 58). A flexible spacer is linked in-frame to the SNAP-25 antigen as a fusion protein.

[037] As discussed above, a flexible spacer is used, in part, to increase the overall peptide length of the SNAP-25 antigen. For example, a 5-10 amino acid SNAP-25 antigen can have its overall length increased by linking a 3-5 amino acid flexible space to the amino-end of the SNAP-25 antigen. As another example, a 5-10 amino acid SNAP-25 antigen can have its overall length increased by linking a 4-6 amino acid flexible space to the amino-end of the SNAP-25 antigen. As another example, a 5-10 amino acid SNAP-25 antigen can have its overall length increased by linking a 7-10 amino acid flexible space to the amino-end of the SNAP-25 antigen. As another example, a 7-12 amino acid SNAP-25 antigen can have its overall length increased by linking a 1-3 amino acid flexible space to the amino-end of the SNAP-25 antigen. As another example, a 7-12 amino acid SNAP-25 antigen can have its overall length increased by linking a 4-6 amino acid flexible space to the amino-end of the SNAP-25 antigen. The increased length provided by the flexible spacer allows for the selection of a small sized SNAP-25 antigen, thereby increasing the likelihood that the SNAP-25 antigen will only trigger an immune response substantially against a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, thus increasing the possibility of producing α-SNAP-25 antibodies that can distinguish a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

[038] It is envisioned that compositions disclosed in the present specification can optionally comprise a SNAP-25 antigen disclosed in the present specification and one or more adjuvants. As used herein, the term "adjuvant" when used in reference to a SNAP-25 composition refers to any substance or mixture of substances that increases or diversifies the immune response to a SNAP-25 antigen. An adjuvant can, for example, serve to reduce the number of immunizations or the amount of antigen required for protective immunization. The use of adjuvants in an immune response inducing composition is well known. The main objective of these adjuvants is to allow an increase in the immune response. Non-limiting adjuvants include, *e.g.*, liposomes, oily phases, including, without limitation, the Freund type of adjuvants, such as, *e.g.*, Freund's complete adjuvant (FCA); Freund's incomplete adjuvant (FIA); sapogenin glycosides, such as, *e.g.*, saponins; carbopol; N-acetylmuramyl-L-alanyl-D-isoglutamine (commonly known as muramyl dipeptide or "MDP"); and lipopolysaccharide (LPS). Such adjuvants are generally used in the form of an emulsion with an aqueous phase, or, more commonly, may consist of water-insoluble inorganic salts. These inorganic salts may consist, for example, of aluminum hydroxide, zinc sulfate, colloidal iron hydroxide, calcium phosphate or calcium chloride. Aluminum hydroxide ($Al(OH)_3$) is a commonly used adjuvant. Currently, the only FDA-approved adjuvant for use in humans is aluminum salts (Alum) which are used to "depot" antigens by precipitation of the antigens. Adjuvants provided above are merely exemplary. In fact, any adjuvant may be used in a SNAP-25 composition disclosed in the present specification as long as the adjuvant satisfies the requisite characteristics for inducing an immune response.

[039] A carrier disclosed in the present specification may also act as an adjuvant. Specific adjuvants and methods of making and using are described in, *e.g.*, Gupta et al. Vaccine, 11: 993-306, 1993; Arnon, R. (Ed.) Synthetic Vaccines 1:83-92, CRC Press, Inc., Boca Raton, Fla., 1987; and David W. Waggoner, Jr. et al., *Immunogenicity-Enhancing Carriers and Compositions Thereof and Methods of Using the Same*, U.S. Patent Publication No. 20040057958 (Mar. 25, 2004). Additional adjuvants include any compound described in Chapter 7 (pp 141-227) of "Vaccine Design, The Subunit and Adjuvant Approach" (eds. Powell, M. F. and Newman, M. J.) Pharmaceutical Biotechnology, Volume 6, Plenum Press (New York). Examples from this compendium include Muramyl Dipeptide (MDP) and Montanide 720. Molecules such as Poly Inosine:Cytosine (Poly I:C) or plasmid DNA containing CpG motifs can also be administered as adjuvants in combination with antigens encapsulated in microparticles. In another example, the adjuvant is an agent that facilitates entry of the antigenic compound into the cytoplasm of a cell such as listeriolysin, streptolysin or a mixture thereof.

[040] Thus, in an embodiment, a SNAP-25 composition comprises a SNAP-25 antigen having a carboxylated carboxyl-terminal glutamine linked to a carrier peptide. In aspects of this embodiment, a SNAP-25 antigen having a carboxylated carboxyl-terminal glutamine comprises SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 147, or SEQ ID NO: 148. In another aspect of this embodiment, a SNAP-25 antigen comprises SEQ ID NO: 38. In

aspects of this embodiment, the carrier peptide is a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI) or a multiple attachment peptide (MAP).

[041] In another embodiment, a SNAP-25 composition comprises a SNAP-25 antigen having a carboxylated carboxyl-terminal lysine linked to a carrier peptide. In aspects of this embodiment, SNAP-25 antigen having a carboxylated carboxyl-terminal lysine comprises SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44. In another aspect of this embodiment, a SNAP-25 antigen comprises SEQ ID NO: 45. In aspects of this embodiment, the carrier peptide is a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI) or a multiple attachment peptide (MAP).

[042] In yet another embodiment, a SNAP-25 composition comprises a SNAP-25 antigen having a carboxylated C-terminal glutamine linked to one or more flexible linkers and a carrier peptide wherein the flexible linkers intervene between the SNAP-25 antigen and the carrier peptide. In aspects of this embodiment, SNAP-25 antigen having a carboxylated carboxyl-terminal glutamine comprises SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 147, or SEQ ID NO: 148. In another embodiment, a SNAP-25 antigen comprises SEQ ID NO: 46. In aspects of this embodiment, the carrier peptide is a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI) or a multiple attachment peptide (MAP). In aspects of this embodiment, the flexible linker is a G-spacer or an A-spacer.

[043] In still another embodiment, a SNAP-25 composition comprises a SNAP-25 antigen having a carboxylated C-terminal lysine linked to a flexible linker and a carrier peptide wherein the flexible linker intervenes between the SNAP-25 antigen and the carrier peptide. In aspects of this embodiment, SNAP-25 antigen having a carboxylated carboxyl-terminal lysine comprises SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44. In another aspect of this embodiment, a SNAP-25 antigen comprises SEQ ID NO: 47. In aspects of this embodiment, the carrier peptide is a keyhole limpet hemacyanin (KLH), an ovalbumin (OVA), a thyroglobulin (THY), a bovine serum albumin (BSA), a soybean trypsin inhibitor (STI) or a multiple attachment peptide (MAP). In aspects of this embodiment, the flexible linker is a G-spacer or an A-spacer.

[044] Aspects of the present disclosure comprise, in part, a method for producing α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. An α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be produced by a wide variety of methods that are well known in the art. Specific protocols for making and using antibodies as well as detecting, and measuring antibody

binding specificity, binding affinity and binding avidity are known in the art. *See, e.g.*, ANTIBODIES: A LABORATORY MANUAL (Edward Harlow & David Lane, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1998a); and USING ANTIBODIES: A LABORATORY MANUAL: PORTABLE PROTOCOL No. I (Edward Harlow & David Lane, Cold Spring Harbor Laboratory Press, 1998b); Molecular Cloning, A Laboratory Manual, 2001; and Current Protocols in Molecular Biology, 2004; David Anderson et al., *Therapeutic Polypeptides, Nucleic Acids Encoding Same, and Methods of Use*, U.S. Patent 7,034,132 (Apr. 25, 2005); and Beatriz M. Carreno et al., *Antibodies Against CTLA4*, U.S. Patent 7,034,121 (Apr. 25, 2006).

[045] As a non-limiting example, α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be produced by injecting an animal, such as, *e.g.*, a rabbit, a goat, a mouse or another mammal, with one or more injections of a composition disclosed in the present specification. As another non-limiting example, α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be produced by injecting an egg, such as, *e.g.*, a chicken egg, with one or more injections of a composition disclosed in the present specification. The antibody titer in the immunized animal can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized antigen or a cell-based activity assay. If desired, polyclonal antibodies for an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A affinity chromatography to obtain the IgG fraction, or by affinity purification against the peptide used for producing the antibodies.

[046] As another non-limiting example, α-SNAP-25 monoclonal antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be produced using a hybridoma method. See e.g., Chapter 6 *Monoclonal Antibodies*, pp. 196-244, Harlow & Lane, *supra*, 1998a; and Chapter 7 *Growing Hybridomas*, pp. 245-282, Harlow & Lane, *supra*, 1998a; and Goding, pp. 59-103, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986). In this method, a host animal, such as, e.g., a mouse, a hamster, or another appropriate host animal, is typically exposed to one or more injections of a SNAP-25 antigen disclosed in the present specification to elicit lymphocytes that produce or are capable of producing α-SNAP-25 antibodies that will specifically bind to a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. The antibody titer in the immunized animal can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized antigen or a cell-based activity assay. Alternatively, the lymphocytes can be immunized in vitro using a suitable cell culture line. At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells are isolated from the animal. Generally, either peripheral blood lymphocytes are used, if cells of human origin are desired, or spleen cells or lymph node cells are

used, if non-human mammalian sources are desired. The isolated antibody-producing cells are fused with an immortal cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Typically, a murine myeloma cell line is fused with splenocytes harvested from an appropriately immunized mouse to produce the hybridoma. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine (HAT). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days in culture because they are not transformed). The culture medium in which the hybridoma cells are grown can then be assayed for the presence of α-SNAP-25 monoclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. For example, hybridoma supernatants can be screened using α-SNAP-25 positive media in an immunoprecipitation assay, in vitro binding assay, such as, *e.g.*, a radioimmunoassay (RIA) or an enzyme-linked immunoabsorbent assay (ELISA), or in a cell-based activity assay. Such techniques and assays are known in the art. See e.g., Chapter 11 *Immunoprecipitation*, pp. 421-470, Harlow & Lane, *supra*, 1998a; Chapter 12 *Immunoblotting*, pp. 471-510, Harlow & Lane, *supra*, 1998a; Chapter 14 *Immunoassays*, pp. 553-612, Harlow & Lane, *supra*, 1998a. Additional studies can then be done to determine whether the antibody is also unreactive to a SNAP-25 lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. The binding affinity of an α-SNAP-25 monoclonal antibody can also be determined, *e.g.*, by Scatchard analysis. *See, e.g.*, Peter J. Munson and David Rodbard, *Ligand: A Versatile Computerized Approach For Characterization of Ligand-Binding Systems*, 107(1) Anal. Biochem. 220-239 (1980). After the desired hybridoma cells are identified, limiting dilution procedures are used to isolate clones originating from a single cell until a clonal cell line expressing the desired monoclonal antibody is obtained. Those antibodies sufficiently selective for a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond and bind with sufficiently high avidity are chosen for further characterization and study.

[047] Another alternative for preparing an α-SNAP-25 monoclonal antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product is by screening a recombinant combinatorial immunoglobulin library, such as, *e.g.*, an antibody phage display library, with a SNAP-25 peptide and isolate immunoglobulin library members that bind a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. Kits for generating and screening phage display libraries are commercially available, such as, e.g., the Recombinant Phage Antibody System (Amersham GE Healthcare, Piscataway, NJ); and the SurfZAP™ Phage Display Kit (Stratagene, La Jolla, CA). Additionally, examples of methods and reagents useful in generating and screening antibody display library can be found in, for example, Ladner et al. U.S. Patent 5,223,409; Borrebaeck et al. U.S. Patent 5,712,089; Griffiths et al. U.S. Patent

5,885,793; Griffiths et al. U.S. Patent 5,962,255; McCafferty et al. U.S. Patent 5,969,108; Griffiths et al. U.S. Patent 6,010,884; Jespers et al. U.S. Patent 6,017,732; Borrebaeck et al. U.S. Patent 6,027,930; Johnson et al. U.S. Patent 6,140,471; McCafferty et al. U.S. Patent 6,172,197, each of which is hereby incorporated by reference in its entirety.

[048] Aspects of the present disclosure comprise, in part, collecting a sample containing an α-SNAP-25 antibody or α-SNAP-25 antibody-producing cells. As used herein, the term "sample containing an α-SNAP-25 antibody or α-SNAP-25 antibody-producing cell" refers to any biological matter that contains or potentially contains at least one an α-SNAP-25 antibody that that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. It is envisioned that any and all samples that can contain an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be used in this method, including, without limitation, blood, plasma, serum and lymph fluid. It is also envisioned that any cell capable of producing an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be used in this method, including, without limitation, a CD8 cells, a CTL cell, a helper T-cell and a B-cell. A variety of well known methods can be used for collecting from an individual a sample containing the α-SNAP-25 antibody or α-SNAP-25 antibody-producing cell, see, *e.g.*, Harlow & Lane, *supra,* 1998a; and Harlow & Lane, *supra,* 1998b. Similarly, a variety of well known methods can be used for processing a sample to isolate an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. A procedure for collecting a sample can be selected based on the type of antibody to be isolated. As a non-limiting example, when isolating an α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, an appropriate sample can be a blood sample containing such α-SNAP-25 antibodies, whereas when isolating an α-SNAP-25 monoclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, an appropriate sample can be an α-SNAP-25 antibody-producing cell such as a spleen cell or hybridoma.

[049] Aspects of the present disclosure comprise, in part, isolating an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product from the sample. Methods of isolating an such α-SNAP-25 antibodies, such as, *e.g.*, α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product or α-SNAP-25 monoclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product are well known to those skilled in the art. See, *e.g.*, Harlow and Lane, supra, 1998a; and Harlow and Lane, supra, 1998b. For example, such α-SNAP-25 polyclonal antibodies can be isolated from the sample by well known

techniques, such as, *e.g.*, affinity chromatography using protein A or protein G, which provide primarily the IgG fraction of immune serum. Subsequently, or alternatively, a specific SNAP-25 antigen can be immobilized on a column or magnetic beads to purify the α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product by immunoaffinity chromatography. An α-SNAP-25 monoclonal antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product can be isolated from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, *e.g.*, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[050] Thus, in an embodiment, a method of producing an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product comprises the steps (a) administering to an animal a composition comprising a SNAP-25 antigen having a carboxylated C-terminal glutamine linked to a carrier peptide; (b) collecting from the animal a sample containing an α-SNAP-25 antibody or α-SNAP-25 antibody-producing cell; and (c) isolating the α-SNAP-25 antibody component from the sample. In an aspect of this embodiment, the α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product is a polyclonal antibody. In another aspect of this embodiment, an α-SNAP-25 antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product is a monoclonal antibody. In a further aspect of this embodiment, an α-SNAP-25 monoclonal antibody that binds an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product produced is an IgG subtype. In other aspects of this embodiment, SNAP-25 composition further comprises an adjuvant, such as, *e.g.*, polyethylene glycol (PEG), monomethoxypolyethylene glycol (mPEG), or polyvinyl alcohol (PVA).

[051] In another embodiment, a method of producing α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product comprises the steps (a) administering to an animal a composition comprising a SNAP-25 peptide having a carboxylated C-terminal glutamine linked to a flexible linker and a carrier peptide wherein the flexible linker intervenes between the SNAP-25 peptide and the carrier peptide; (b) collecting from the animal a sample containing an α-SNAP-25 antibody or α-SNAP-25 antibody-producing cell; and (c) isolating the α-SNAP-25 antibody from the sample. In an aspect of this embodiment, the α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product is a polyclonal antibody. In another aspect of this embodiment, α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product is a monoclonal antibody. In a further aspect of this embodiment, an α-SNAP-25 monoclonal antibody that binds an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A

cleavage site scissile bond from a SNAP-25 cleavage product produced in an IgG subtype. In other aspects of this embodiment, SNAP-25 composition further comprises an adjuvant, such as, *e.g.*, polyethylene glycol (PEG), monomethoxypolyethylene glycol (mPEG), or polyvinyl alcohol (PVA).

[052] Aspects of the present disclosure comprise, in part, an isolated α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. As used herein, the term "isolated" refers to separating a molecule from its natural environment by the use of human intervention. As used herein, the term "antibody" refers to a molecule generated by an immune system that was made in response to a particular antigen that specifically binds to that antigen, and includes both naturally occurring antibodies and non-naturally occurring antibodies. As used herein, the term "α-SNAP-25" is synomonous with "anti-SNAP-25" and refers to an antibody that binds to a SNAP-25 antigen. For example, an antibody can be a polyclonal antibody, a monoclonal antibody, a dimer, a multimer, a multispecific antibody, a humanized antibody, a chimeric antibody, bi-functional antibody, a cell-associated antibody like an Ig receptor, a linear antibody, a diabody, or a minibody, so long as the fragment exhibits the desired biological activity, and single chain derivatives of the same. An antibody can be a full-length immunoglobulin molecule comprising the V_H and V_L domains, as well as a light chain constant domain (C_L) and heavy chain constant domains, C_{H1}, C_{H2} and C_{H3}, or an immunologically active fragment of a full-length immunoglobulin molecule, such as, *e.g.*, a Fab fragment, a F(ab')$_2$ fragment, a Fc fragment, a Fd fragment, a Fv fragment. An antibody can be derived from any vertebrate species (*e.g.*, human, goat, horse, donkey, murine, rat, rabbit, or chicken), and can be of any type (*e.g.*, IgG, IgE, IgM, IgD, and IgA), class (*e.g.*, IgA, IgD, IgE, IgG, and IgM) or subclass (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). For general disclosure on the structure of naturally occurring antibodies, non-naturally occurring antibodies, and antigenic compound-binding fragments thereof, see, *e.g.*, Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995), each of which is hereby incorporated by reference in its entirety.

[053] Naturally-occurring antibodies are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

[054] The complete antigen-recognition and antigen-binding site is contained within the variable domains of the antibody, *i.e.*, the Fv fragment. This fragment includes a dimer of one heavy chain variable domain (V_H) and one light chain variable domain (V_L) in tight, non-covalent association. Each domain comprises four framework regions (FR), which largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases form part of, the β-sheet structure. Each hypervariable region comprises an amino acid sequence corresponding to a complementarity determining region (CDRs). Collectively, it the three-dimensional configuration of the six CDR regions that define an antigen-binding site on the surface of the V_H–V_L dimmer that confers antigen-binding specificity. *See e.g.*, Cyrus Chothia, et al., *Conformations of Immunoglobulin Hypervariable Regions*, Nature 342(6252): 877-883 (1989); Elvin A. Kabat, et al *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), each of which is incorporated by reference in its entirety. The constant domains of the antibody are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

[055] A target antigen generally has one or more binding sites, also called epitopes, which are recognized by the CDR-formed antigen-binding site. As used herein, an "epitope" is synonymous with "antigenic determinant" and refers to the site on a target antigen, such as, *e.g.*, a peptide, polysaccharide or lipid-containing molecule, capable of specific binding to an immunoglobulin or T-cell receptor or otherwise interacting with a molecule. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may have more than one corresponding antibody.

[056] Polyclonal antibodies refer to a heterogeneous population of antibody molecules that contain at least two species of antibody capable of binding to a particular antigen. By definition, a polyclonal antibody includes two different antibodies that bind to at least two different epitopes. As used herein, the term "monoclonal antibody" or "monoclonal antibodies" refer to a substantially homogeneous population of antibody molecules that contain only one species of antibody capable of binding a particular antigen *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. By definition, a monoclonal antibody binds to a single epitope. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibodies, each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al (1975) Nature 256:495, or may be made by recombinant DNA methods (see for example: U.S. Pat. No. 4,816,567; U.S. Pat. No. 5,807,715). The

monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in Clackson et al (1991) Nature, 352:624-628; Marks et al (1991) J. Mol. Biol., 222:581-597; for example.

[057] Thus, in an embodiment, an α-SNAP-25 antibody comprises a heavy chain variable domain (V_H) and a light chain variable domain (V_L) that selectively binds to a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In an aspect of this embodiment, the heavy chain variable domain (V_H) is SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 80, or SEQ ID NO: 82. In another aspect of this embodiment, the light chain variable domain (V_L) is SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, or SEQ ID NO: 92.

[058] In another embodiment, an α-SNAP-25 antibody comprises a heavy chain variable domain (V_H) CDR1 region, a CDR2 region, a CDR3 region, or any combination thereof that selectively binds to a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In an aspect of this embodiment, the heavy chain variable domain (V_H) CDR1 region is SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 118, SEQ ID NO: 119, or SEQ ID NO: 120. In another aspect of this embodiment, the heavy chain variable domain (V_H) CDR2 region is SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 121, SEQ ID NO: 122, or SEQ ID NO: 123. In yet another aspect of this embodiment, the heavy chain variable domain (V_H) CDR3 region is SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, or SEQ ID NO: 124.

[059] In another embodiment, an α-SNAP-25 antibody comprises a light chain variable domain (V_L) CDR1 region, a CDR2 region, a CDR3 region, or any combination thereof that selectively binds to a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In an aspect of this embodiment, the light chain variable domain (V_L) CDR1 region is SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, or SEQ ID NO: 129. In another aspect of this embodiment, the light chain variable domain (V_L) CDR2 region is SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, or SEQ ID NO: 112. In yet another aspect of this embodiment, the light chain variable domain (V_L) CDR3 region is SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, or SEQ ID NO: 117.

[060] In yet another embodiment, an α-SNAP-25 antibody specifically binds an epitope comprising a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In an aspect of this embodiment, the epitope comprises SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 147, or SEQ ID NO: 148. In an aspect of this embodiment, the epitope comprises SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

[061] As discussed above, the sequence surrounding a BoNT/A cleavage site present in SNAP-25 is denoted P_5–P_4–P_3–P_2–P_1–P_1'–P_2'–P_3'–P_4'–P_5', with P_1–P_1' representing the scissile bond. Upon cleavage by BoNT/A, the resulting cleavage products produced comprise a fragment including the P_5–P_4–P_3–P_2–P_1 sequence and a fragment including the P_1'–P_2'–P_3'–P_4'–P_5'. As used herein, the term "α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product" refers to α-SNAP-25 antibodies that selectively bind to any SNAP-25 cleavage product fragment comprising the P_5–P_4–P_3–P_2–P_1 sequence, but not to any SNAP-25 cleavage product fragment comprising the P_1'–P_2'–P_3'–P_4'–P_5' sequence or to any SNAP-25 having an intact P_1–P_1' scissile bond of a BoNT/A cleavage site. As used herein, the term "α-SNAP-25_{197} antibody" refers to an antibody that selectively binds to a SNAP-25 having a carboxyl-terminus P_1 residue that corresponds to glutamine 197 of SEQ ID NO: 5. As used herein, the term "α-SNAP-25_{204} antibody" refers to an antibody that selectively binds to a SNAP-25 having a carboxyl-terminus P_1 residue that corresponds to lysine 204 of SEQ ID NO: 16.

[062] As used herein, the term "selectively" refers to having a unique effect or influence or reacting in only one way or with only one thing. As used herein, the term "selectively binds," when made in reference to an antibody, refers to the discriminatory binding of the antibody to the indicated target epitope such that the antibody does not substantially cross react with non-target epitopes. The minimal size of a peptide epitope, as defined herein, is about five amino acids, and a peptide epitope typically comprises at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, or at least 20 amino acids. A peptide epitope may be discontinuous, *i.e.*, it comprises amino acid residues that are not adjacent in the primary structure of the peptide but are brought together into an epitope by way of the secondary, tertiary, or quaternary structure of the peptide. Furthermore, it is also noted that an epitope might comprise a portion of a molecule other than an amino acid sequence, such as, *e.g.*, a carbohydrate moiety, a lipid moiety like lipoproteins or glycolipids, or a chemically-modified amino acid moiety like a phosphorylated amino acid. In aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can selectively bind a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprising at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, or at least 20 amino acids. In other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can selectively bind a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond comprising at most 5, at most 6, at most 7, at most 8, at most 9, at most 10, at most 15, or at most 20 amino acids.

[063] Selective binding includes binding properties such as, *e.g.*, binding affinity, binding specificity, and binding avidity. See David J. King, *Applications and Engineering of Monoclonal Antibodies*, pp. 240 (1998). Binding affinity refers to the length of time the antibody resides at its epitope binding site, and can be viewed as the strength with which an antibody binds its epitope. Binding affinity can be described an

antibody's equilibrium dissociation constant (KD), which is defined as the ratio Kd/Ka at equillibrium. Where Ka is the antibody's association rate constant and kd is the antibody's dissociation rate constant. Binding affinity is determined by both the association and the dissociation and alone neither high association or low dissociation can ensure high affinity. The association rate constant (Ka), or on-rate constant (Kon), measures the number of binding events per unit time, or the propensity of the antibody and the antigen to associate reversibly into its antibody-antigen complex. The association rate constant is expressed in M^{-1} s^{-1}, and is symbolized as follows: [Ab] x [Ag] x Kon. The larger the association rate constant, the more rapidly the antibody binds to its antigen, or the higher the binding affinity between antibody and antigen. The dissociation rate constant (Kd), or off-rate constant (Koff), measures the number of dissociation events per unit time propensity of an antibody-antigen complex to separate (dissociate) reversibly into its component molecules, namely the antibody and the antigen. The dissociation rate constant is expressed in s^{-1}, and is symbolized as follows: [Ab + Ag] x Koff. The smaller the dissociation rate constant, the more tightly bound the antibody is to its antigen, or the higher the binding affinity between antibody and antigen. The equilibrium dissociation constant (KD) measures the rate at which new antibody-antigen complexes formed equals the rate at which antibody-antigen complexes dissociate at equilibrium. The equilibrium dissociation constant is expressed in M, and is defined as Koff/Kon=[Ab] x [Ag]/[Ab + Ag], where [Ab] is the molar concentration of the antibody, [Ag] is the molar concentration of the antigen, and [Ab + Ag] is the of molar concentration of the antibody-antigen complex, where all concentrations are of such components when the system is at equilibrium. The smaller the equilibrium dissociation constant, the more tightly bound the antibody is to its antigen, or the higher the binding affinity between antibody and antigen.

[064] Thus, in an embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an association rate constant of, *e.g.*, less than 1 x 10^5 M^{-1} s^{-1}, less than 1 x 10^6 M^{-1} s^{-1}, less than 1 x 10^7 M^{-1} s^{-1}, or less than 1 x 10^8 M^{-1} s^{-1}. In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an association rate constant of, *e.g.*, more than 1 x 10^5 M^{-1} s^{-1}, more than 1 x 10^6 M^{-1} s^{-1}, more than 1 x 10^7 M^{-1} s^{-1}, or more than 1 x 10^8 M^{-1} s^{-1}. In other aspects, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an association rate constant between 1 x 10^5 M^{-1} s^{-1} to 1 x 10^8 M^{-1} s^{-1}, 1 x 10^6 M^{-1} s^{-1} to 1 x 10^8 M^{-1} s^{-1}, 1 x 10^5 M^{-1} s^{-1} to 1 x 10^7 M^{-1} s^{-1}, or 1 x 10^6 M^{-1} s^{-1} to 1 x 10^7 M^{-1} s^{-1}.

[065] In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have a disassociation rate constant of less than 1 x 10^{-3} s^{-1}, less than 1 x 10^{-4} s^{-1}, or less than 1 x 10^{-5} s^{-1}. In other aspects of this embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site

scissile bond can have a disassociation rate constant of, *e.g.*, less than 1.0 x 10^{-4} s^{-1}, less than 2.0 x 10^{-4} s^{-1}, less than 3.0 x 10^{-4} s^{-1}, less than 4.0 x 10^{-4} s^{-1}, less than 5.0 x 10^{-4} s^{-1}, less than 6.0 x 10^{-4} s^{-1}, less than 7.0 x 10^{-4} s^{-1}, less than 8.0 x 10^{-4} s^{-1}, or less than 9.0 x 10^{-4} s^{-1}. In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have a disassociation rate constant of, *e.g.*, more than 1 x 10^{-3} s^{-1}, more than 1 x 10^{-4} s^{-1}, or more than 1 x 10^{-5} s^{-1}. In other aspects of this embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have a disassociation rate constant of, *e.g.*, more than 1.0 x 10^{-4} s^{-1}, more than 2.0 x 10^{-4} s^{-1}, more than 3.0 x 10^{-4} s^{-1}, more than 4.0 x 10^{-4} s^{-1}, more than 5.0 x 10^{-4} s^{-1}, more than 6.0 x 10^{-4} s^{-1}, more than 7.0 x 10^{-4} s^{-1}, more than 8.0 x 10^{-4} s^{-1}, or more than 9.0 x 10^{-4} s^{-1}.

[066] In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an equilibrium disassociation constant of less than 0.500 nM. In aspects of this embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an equilibrium disassociation constant of, *e.g.*, less than 0.500 nM, less than 0.450 nM, less than 0.400 nM, less than 0.350 nM, less than 0.300 nM, less than 0.250 nM, less than 0.200 nM, less than 0.150 nM, less than 0.100 nM, or less than 0.050 nM. In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an equilibrium disassociation constant of more than 0.500 nM. In aspects of this embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an equilibrium disassociation constant of, *e.g.*, more than 0.500 nM, more than 0.450 nM, more than 0.400 nM, more than 0.350 nM, more than 0.300 nM, more than 0.250 nM, more than 0.200 nM, more than 0.150 nM, more than 0.100 nM, or more than 0.050 nM.

[067] In yet another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an association rate constant of for the intact SNAP-25 of, *e.g.*, less than 1 x 10^{0} M^{-1} s^{-1}, less than 1 x 10^{1} M^{-1} s^{-1}, less than 1 x 10^{2} M^{-1} s^{-1}, less than 1 x 10^{3} M^{-1} s^{-1}, or less than 1 x 10^{4} M^{-1} s^{-1}. In another embodiment, the binding affinity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can have an association rate constant of for the intact SNAP-25 of, *e.g.*, at most 1 x 10^{0} M^{-1} s^{-1}, at most 1 x 10^{1} M^{-1} s^{-1}, at most 1 x 10^{2} M^{-1} s^{-1}, at most 1 x 10^{3} M^{-1} s^{-1}, or at most 1 x 10^{4} M^{-1} s^{-1}.

[068] Binding specificity is the ability of an antibody to discriminate between a molecule containing its epitope and a molecule that does not contain that epitope. One way to measure binding specificity is to

compare the Kon association rate of the antibody for a molecule containing its epitope relative to the Kon association rate of the antibody for a molecule that does not contain that epitope. For example, comparing the association rate constant (Ka) of an α-SNAP-25 antibody for a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond relative to a SNAP-25 not comprising that epitope, such as, *e.g.*, a SNAP-25 epitope lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond or a SNAP-25 epitope having an intact P_1–P_1' scissile bond of a BoNT/A cleavage site. In aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for a SNAP-25 not comprising its epitope(s) of, *e.g.*, less than 1 x 10^0 M^{-1} s^{-1}, less than 1 x 10^1 M^{-1} s^{-1}, less than 1 x 10^2 M^{-1} s^{-1}, less than 1 x 10^3 M^{-1} s^{-1} or less than 1 x 10^4 M^{-1} s^{-1}. In other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for a SNAP-25 not comprising its epitope(s) of, *e.g.*, at most 1 x 10^0 M^{-1} s^{-1}, at most 1 x 10^1 M^{-1} s^{-1}, at most 1 x 10^2 M^{-1} s^{-1}, at most 1 x 10^3 M^{-1} s^{-1} or at most 1 x 10^4 M^{-1} s^{-1}.

[069] In yet aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for its epitope relative to a SNAP-25 not comprising that epitope of, *e.g.*, at least 2-fold more, at least 3-fold more, at least 4-fold more, at least 5-fold more, at least 6-fold more, at least 7-fold more, at least 8-fold more, or at least 9-fold more. In further aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for its epitope relative to a SNAP-25 not comprising that epitope of, *e.g.*, at least 10-fold more, at least 100-fold more, at least 1,000-fold more or at least 10,000-fold more. In yet other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for its epitope relative to a SNAP-25 not comprising that epitope of, *e.g.*, at most 1-fold more, at most 2-fold more, at most 3-fold more, at most 4-fold more, at most 5-fold more, at most 6-fold more, at most 7-fold more, at most 8-fold more, or at most 9-fold more. In yet other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has an association rate constant (Ka) for its epitope relative to a SNAP-25 not comprising that epitope of, *e.g.*, at most 10-fold more, at most 100-fold more, at most 1,000-fold more or at most 10,000-fold more.

[070] The binding specificity of an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can also be characterized as a ratio that such an α-SNAP-25 antibody can discriminate its SNAP-25 epitope relative to a SNAP-25 not comprising that epitope, such as, *e.g.*, a SNAP-25 epitope lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond or a SNAP-25 epitope having an intact P_1–P_1'

scissile bond of a BoNT/A cleavage site. In aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has a binding specificity ratio for its SNAP-25 epitope relative to a SNAP-25 not comprising that epitope of, *e.g.*, at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 64:1, at least 7:1, at least 8:1, at least 9:1, at least 10:1, at least 15:1, at least 20:1, at least 25:1, at least 30:1, at least 35:1, or at least 40:1. In yet other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has a binding specificity ratio for its SNAP-25 epitope relative to a SNAP-25 lacking a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond of, *e.g.*, at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 6:1, at least 7:1, at least 8:1, at least 9:1, at least 10:1, at least 15:1, at least 20:1, at least 25:1, at least 30:1, at least 35:1, or at least 40:1. In still other aspects of this embodiment, an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond has a binding specificity ratio for its SNAP-25 epitope relative to a SNAP-25 having an intake P_1–P_1' scissile bond of a BoNT/A cleavage site of, *e.g.*, at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 64:1, at least 7:1, at least 8:1, at least 9:1, at least 10:1, at least 15:1, at least 20:1, at least 25:1, at least 30:1, at least 35:1, or at least 40:1.

[071] Binding avidity, also known as functional affinity, refers to the sum total of the functional binding strength between a multivalent antibody and its antigen. Antibody molecules can have more than one binding site (*e.g.*, 2 for IgG, 10 for IgM), and many antigens contain more than one antigenic site. While binding avidity of an antibody depends on the binding affinities of the individual antibody binding sites, binding avidity is greater than the binding affinity as all the antibody-antigen interactions must be broken simultaneously for the antibody to dissociate completely. It is envisioned that an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can selectively bind to any and all epitopes for that antibody.

[072] Thus, in an embodiment, an α-SNAP-25 antibody is an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In aspects of this embodiment, an α-SNAP-25 antibody is an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus glutamine or an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus lysine. In other aspects of this embodiment, an α-SNAP-25 antibody is an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus P_1 residue that corresponds to glutamine 197 of SEQ ID NO: 5 or an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus P_1 residue that corresponds to lysine 204 of SEQ ID NO: 16. In still other aspects of this embodiment, an α-SNAP-25 antibody is an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminal amino acid sequence of SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ

ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 147, or SEQ ID NO: 148.

[073] Aspects of the present disclosure comprise, in part, an immuno-based method of detecting BoNT/A activity. The immuno-based methods disclosed in the present specification can be evaluated by several parameters including, *e.g.*, accuracy, precision, limit of detection (LOD), limits of quantitation (LOQ), range, specificity, selectivity, linearity, ruggedness, and system suitability. The accuracy of a method is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted as a conventional true value or an accepted reference value. The precision of a method is the degree of agreement among individual test results, when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. As such, precision evaluates 1) within assay variability; 2) within-day variability (repeatability); and 3) between-day variability (intermediate precision); and 4) between-lab variability (reproducibility). Coefficient of variation (CV%) is a quantitative measure of precision expressed relative to the observed or theoretical mean value.

[074] An immuno-based method disclosed in the present specification must be able to detect, over background, the presence of an α-SNAP-25 antibody-antigen complex comprising a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. The limit of detection (LOD) of a method refers to the concentration of analyte which gives rise to a signal that is significantly different from the negative control or blank and represents the lowest concentration of analyte that can be distinguished from background.

[075] Thus, in an embodiment, the immuno-based method disclosed in the present specification can detect the LOD of BoNT/A at an amount that is significantly different from a negative control or blank. In aspect of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 10 ng or less, 9 ng or less, 8 ng or less, 7 ng or less, 6 ng or less, 5 ng or less, 4 ng or less, 3 ng or less, 2 ng or less, 1 ng or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 900 pg or less, 800 pg or less, 700 pg or less, 600 pg or less, 500 pg or less, 400 pg or less, 300 pg or less, 200 pg or less, 100 pg or less of a BoNT/A. In further aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 90 pg or less, 80 pg or less, 70 pg or less, 60 pg or less, 50 pg or less, 40 pg or less, 30 pg or less, 20 pg or less, 10 pg or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 9 pg or less, 8 pg or less, 7 pg or less, 6 pg or less, 5 pg or less, 4 pg or less, 3 pg or less, 2 pg or less, 1 pg or less of a BoNT/A. In yet other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 0.9 pg or less, 0.8 pg or less, 0.7 pg or less, 0.6 pg or less, 0.5 pg or less, 0.4 pg or less, 0.3 pg or less, 0.2 pg or less, 0.1 pg or less of a BoNT/A.

[076] In another aspect of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 10 nM or less or less, 9 nM or less, 8 nM or less, 7 nM or less, 6 nM or less, 5 nM or less, 4 nM or less, 3 nM or less, 2 nM or less, or 1 nM or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 900 pM or less, 800 pM or less, 700 pM or less, 600 pM or less, 500 pM or less, 400 pM or less, 300 pM or less, 200 pM or less, or 100 pM or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 100 pM or less, 90 pM or less, 80 pM or less, 70 pM or less, 60 pM or less, 50 pM or less, 40 pM or less, 30 pM or less, 20 pM or less, or 10 pM or less of a BoNT/A. In yet other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 10 pM or less of a BoNT/A, 9 pM or less, 8 pM or less, 7 pM or less, 6 pM or less, 5 pM or less, 4 pM or less, 3 pM or less, 2 pM or less, or 1 pM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 1000 fM or less, 900 fM or less, 800 fM or less, 700 fM or less, 600 fM or less, 500 fM or less, 400 fM or less, 300 fM or less, 200 fM or less, or 100 fM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 100 fM or less, 90 fM or less, 80 fM or less, 70 fM or less, 60 fM or less, 50 fM or less, 40 fM or less, 30 fM or less, 20 fM or less, or 10 fM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOD of, *e.g.*, 10 fM or less, 9 fM or less, 8 fM or less, 7 fM or less, 6 fM or less, 5 fM or less, 4 fM or less, 3 fM or less, 2 fM or less, or 1 fM or less of a botulinum neurotoxin A.

[077] The limits of quantitation (LOQ) are the lowest and the highest concentrations of analyte in a sample or specimen that can be measured with an acceptable level of accuracy and precision. The lower limit of quantitation refers to the lowest dose that a detection method can measure consistently from the background. The upper limit of quantitation is the highest dose that a detection method can measure consistently before saturation of the signal occurs. The linear range of the method is the area between the lower and the upper limits of quantitation. The linear range is calculated by subtracting lower limit of quantitation from the upper limit of quantitation. As used herein, the term "signal to noise ratio for the lower asymptote" refers to the signal detected in the method at the lower limit of detection divided by the background signal. As used herein, the term "signal to noise ratio for the upper asymptote" refers to the signal detected in the method at the upper limit of detection divided by the background signal.

[078] Thus, in an embodiment, the immuno-based method disclosed in the present specification can detect the LOQ of BoNT/A at an amount that is significantly different from a negative control or blank. In aspect of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 10 ng or less, 9 ng or less, 8 ng or less, 7 ng or less, 6 ng or less, 5 ng or less, 4 ng or less, 3 ng or less, 2 ng or less, 1 ng or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 900 pg or less, 800 pg or less, 700 pg or less, 600 pg or less, 500 pg or less, 400 pg or less, 300 pg or less, 200 pg or less, 100 pg or

less of a BoNT/A. In further aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 90 pg or less, 80 pg or less, 70 pg or less, 60 pg or less, 50 pg or less, 40 pg or less, 30 pg or less, 20 pg or less, 10 pg or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 9 pg or less, 8 pg or less, 7 pg or less, 6 pg or less, 5 pg or less, 4 pg or less, 3 pg or less, 2 pg or less, 1 pg or less of a BoNT/A. In yet other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 0.9 pg or less, 0.8 pg or less, 0.7 pg or less, 0.6 pg or less, 0.5 pg or less, 0.4 pg or less, 0.3 pg or less, 0.2 pg or less, 0.1 pg or less of a BoNT/A.

[079] In another aspect of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 10 nM or less, 9 nM or less, 8 nM or less, 7 nM or less, 6 nM or less, 5 nM or less, 4 nM or less, 3 nM or less, 2 nM or less, or 1 nM or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 900 pM or less, 800 pM or less, 700 pM or less, 600 pM or less, 500 pM or less, 400 pM or less, 300 pM or less, 200 pM or less, or 100 pM or less of a BoNT/A. In other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 100 pM or less, 90 pM or less, 80 pM or less, 70 pM or less, 60 pM or less, 50 pM or less, 40 pM or less, 30 pM or less, 20 pM or less, or 10 pM or less of a BoNT/A. In yet other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 10 pM or less of a BoNT/A, 9 pM or less, 8 pM or less, 7 pM or less, 6 pM or less, 5 pM or less, 4 pM or less, 3 pM or less, 2 pM or less, or 1 pM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 1000 fM or less, 900 fM or less, 800 fM or less, 700 fM or less, 600 fM or less, 500 fM or less, 400 fM or less, 300 fM or less, 200 fM or less, or 100 fM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 100 fM or less, 90 fM or less, 80 fM or less, 70 fM or less, 60 fM or less, 50 fM or less, 40 fM or less, 30 fM or less, 20 fM or less, or 10 fM or less of a BoNT/A. In still other aspects of this embodiment, the immuno-based method disclosed in the present specification has an LOQ of, *e.g.*, 10 fM or less, 9 fM or less, 8 fM or less, 7 fM or less, 6 fM or less, 5 fM or less, 4 fM or less, 3 fM or less, 2 fM or less, or 1 fM or less of a BoNT/A.

[080] An immuno-based assay useful to practice aspect of the disclosed methods must have a precision of no more than 50%. In aspects of this embodiment, an immuno-based assay has a precision of no more than 50%, no more than 40%, no more than 30%, or no more than 20%. In other aspects of this embodiment, an immuno-based assay has a precision of nor more than 15%, no more than 10%, or no more than 5%. In other aspects of this embodiment, an immuno-based assay has a precision of nor more than 4%, no more than 3%, no more than 2%, or no more than 1%.

[081] An immuno-based assay useful to practice aspect of the disclosed methods must have an accuracy of at least 50%. In aspects of this embodiment, an immuno-based assay has an accuracy of at

least 50%, at least 60%, at least 70%, or at least 80%. In other aspects of this embodiment, an immuno-based assay has an accuracy of at least 85%, at least 90%, or at least 95%. In other aspects of this embodiment, an immuno-based assay has an accuracy of at least 96%, at least 97%, at least 98%, or at least 99%.

[082] An immuno-based method disclosed in the present specification must have a signal to noise ratio for the lower asymptote that is statistically significant and a signal to noise ratio for the upper asymptote that is statistically significant. In aspects of this embodiment, an immuno-based method disclosed in the present specification has a signal to noise ratio for the lower asymptote of, *e.g.*, at least 3:1, at least 4:1,at least 5:1, at least 6:1, at least 7:1, at least 8:1, at least 9:1, at least 10:1, at least 15:1 or at least 20:1. In other aspects of this embodiment, an immuno-based method has a signal to noise ratio for the upper asymptote of, *e.g.*, at least 10:1, at least 15:1, at least 20:1, at least 25:1, at least 30:1, at least 35:1, at least 40:1, at least 45:1, at least 50:1, at least 60:1, at least 70:1, at least 80:1, at least 90:1, or at least 100:1, at least 150:1, at least 200:1, at least 250:1, at least 300:1, at least 350:1, at least 400:1, at least 450:1, at least 500:1, at least 550:1, or at least 600:1.

[083] The specificity of a method defines the ability of the method to measure the analyte of interest to the exclusion of other relevant components, such as, *e.g.*, partially-active or inactive analyte. The selectivity of a method describes the ability of an analytical method to differentiate various substances in a sample. The linearity of a method is its ability to elicit results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in the sample. Thus in an embodiment, an immuno-based method disclosed in the present specification can distinguish a fully-active BoNT/A from a partially-active BoNT/A having, *e.g.*, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, or 10% or less the activity of a fully-active BoNT/A.

[084] The ruggedness of the method is the reproducibility of the test results obtained for identical samples under normal (but variable) test conditions. Robustness of a procedure is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability in normal usage. Thus, whereas ruggedness evaluates unavoidable changes, robustness evaluates deliberate changes. Typical parameters evaluated by ruggedness and robustness include the effects of freeze/thaw, incubation times, incubation temperature, longevity of reagent, sample preparation, sample storage, cell passage number, lots of toxin, variability between purifications, and variability between nicking reactions. Robustness parameters for cell-based assays include the cell bank (beginning, middle and end of freeze), cell passage level, cell seeding density, cell stock density (how many days in culture), cell age in flask (waiting time to seeding), incubation time, different plates, excessive amounts of serum, and source of reagents. The system suitability of the method is the determination of assay performance, including the performance of reagents and instruments, over time by analysis of a reference standard. System suitability is stressed in FDA guidance referring to the fact that equipment, electronics, assay performance, and samples to be

analyzed, constitute an integrated system. System suitability can be evaluated by testing for parallelism, which is when plotting the log dose versus the response, serial dilutions of the reference and serial dilutions of the samples should give rise to parallel curves.

[085] Aspects of the present disclosure comprise, in part, a cell from an established cell line. As used herein, the term "cell" refers to any eukaryotic cell susceptible to BoNT/A intoxication by a BoNT/A or any eukaryotic cell that can uptake a BoNT/A. The term cell encompasses cells from a variety of organisms, such as, *e.g.*, murine, rat, porcine, bovine, equine, primate and human cells; from a variety of cell types such as, *e.g.*, neuronal and non-neuronal; and can be isolated from or part of a heterogeneous cell population, tissue or organism. As used herein, the term "established cell line" is synonymous with "immortal cell line," or "transformed cell line" and refers to a cell culture of cells selected for indefinite propagation from a cell population derived from an organism, tissue, or organ source. By definition, an established cell line excludes a cell culture of primary cells. As used herein, the term "primary cells" are cells harvested directly from fresh tissues or organs and do not have the potential to propagate indefinitely. An established cell line can comprise a heterogeneous population of cells or a uniform population of cells. An established cell line derived from a single cell is referred to as a clonal cell line. An established cell line can be one whose cells endogenously express all component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate and encompasses the binding of a BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the BoNT/A light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a SNAP-25. Alternatively, an established cell line can be one whose cells have had introduced from an exogenous source at least one component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate and encompasses the binding of a BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the BoNT/A light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a SNAP-25. Also refereed to as a genetically-engineered cell line, cells from such an established cell line may, *e.g.*, express an exogenous FGFR2, an exogenous FGFR3, an exogenous SV2, an exogenous SNAP-25, or any combination thereof.

[086] Aspects of the present disclosure comprise, in part, a cell from an established cell line susceptible to BoNT/A intoxication. As used herein, the terms "cell(s) susceptible to BoNT/A intoxication," "cell(s) susceptible to BoNT/A intoxication by a BoNT/A," or "cell(s) from an established cell line susceptible to BoNT/A intoxication by a BoNT/A" refer to cell(s) that can undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate and encompasses the binding of a BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the BoNT/A light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a SNAP-25. By definition, cell(s) susceptible to of BoNT/A intoxication must express, or be engineered to express, at least one BoNT/A receptor and at least one SNAP-25 substrate. As used herein, the terms "cell(s) that can uptake BoNT/A" or "cell(s) comprising an established cell line that can uptake BoNT/A"

refer to cells that can undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate and encompasses the binding of a BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the BoNT/A light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a SNAP-25. By definition, cell(s) that can uptake BoNT/A must express, or be engineered to express, at least one BoNT/A receptor and at least one SNAP-25 substrate.

[087] Thus in an embodiment, cells from an established cell line are susceptible to BoNT/A intoxication. In aspects of this embodiment, cells from an established cell line are susceptible to BoNT/A intoxication by, *e.g.*, about 500 pM or less, about 400 pM or less, about 300 pM or less, about 200 pM or less, or about 100 pM or less of a BoNT/A. In other aspects of this embodiment, cells from an established cell line are susceptible to BoNT/A intoxication by, *e.g.*, about 90 pM or less, about 80 pM or less, about 70 pM or less, about 60 pM or less, about 50 pM or less, about 40 pM or less, about 30 pM or less, about 20 pM or less A, or about 10 pM or less of a BoNT/A. In still other aspects, cells from an established cell line are susceptible to BoNT/A intoxication by, *e.g.*, about 9 pM or less, about 8 pM or less, about 7 pM or less, about 6 pM or less, about 5 pM or less, about 4 pM or less, about 3 pM or less, about 2 pM or less, or about 1 pM or less of a BoNT/A. In yet other aspects, cells from an established cell line are susceptible to BoNT/A intoxication by, *e.g.*, about 0.9 pM or less, about 0.8 pM or less, about 0.7 pM or less, about 0.6 pM or less, about 0.5 pM or less, about 0.4 pM or less, about 0.3 pM or less, about 0.2 pM, or about 0.1 pM or less of a BoNT/A. As used herein, the term "about" when qualifying a value of a stated item, number, percentage, or term refers to a range of plus or minus ten percent of the value of the stated item, percentage, parameter, or term.

[088] In another embodiment, cells comprising an established cell line can uptake a BoNT/A. In aspects of this embodiment, cells comprising an established cell line can uptake, *e.g.*, about 500 pM or less, about 400 pM or less, about 300 pM or less, about 200 pM or less, or about 100 pM or less of a BoNT/A. In other aspects of this embodiment, cells comprising an established cell line possess the ability to uptake about 90 pM or less, about 80 pM or less, about 70 pM or less, about 60 pM or less, about 50 pM or less, about 40 pM or less, about 30 pM or less, about 20 pM or less, or about 10 pM or less of a BoNT/A. In still other aspects, cells comprising an established cell line possess the ability to uptake about 9 pM or less, about 8 pM or less, about 7 pM or less, about 6 pM or less, about 5 pM or less, about 4 pM or less, about 3 pM or less, about 2 pM or less, or about 1 pM or less of a BoNT/A. In yet other aspects, cells comprising an established cell line possess the ability to uptake about 0.9 pM or less, about 0.8 pM or less, about 0.7 pM or less, about 0.6 pM or less, about 0.5 pM or less, about 0.4 pM or less, about 0.3 pM or less, about 0.2 pM or less, or about 0.1 pM or less of a BoNT/A.

[089] Aspects of the present disclosure comprise, in part, a BoNT/A. As used herein, the term "BoNT/A" is synonymous with "botulinum neurotoxin serotype A" or "botulinum neurotoxin type A" and refers to both a naturally-occurring BoNT/A or a non-naturally occurring BoNT/As thereof, and includes

BoNT/A complex comprising the about 150 kDa BoNT/A neurotoxin and associated non-toxin associated proteins (NAPs), as well as the about 150 kDa BoNT/A neurotoxin alone. Non-limiting examples of BoNT/A complexes include, *e.g.*, the 900-kDa BoNT/A complex, the 500-kDa BoNT/A complex, the 300-kDa BoNT/A complex. Non-limiting examples of the about 150 kDa BoNT/A neurotoxin include, *e.g.*, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4.

[090] As used herein, the term "naturally occurring BoNT/A" refers to any BoNT/A produced by a naturally-occurring process, including, without limitation, BoNT/A isoforms produced from a post-translational modification, an alternatively-spliced transcript, or a spontaneous mutation, and BoNT/A subtypes, such as, *e.g.*, a BoNT/A1 subtype, BoNT/A2 subtype, BoNT/A3 subtype, BoNT/A4 subtype, and BoNT/A5 subtype. A naturally occurring BoNT/A includes, without limitation, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or one that substitutes, deletes or adds, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 amino acids from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. Commercially available pharmaceutical compositions of a naturally-occurring BoNT/A includes, without limitation, BOTOX® (Allergan, Inc., Irvine, CA), DYSPORT®/RELOXIN®, (Ipsen Ltd., Slough, England), PURTOX® (Mentor Corp., Santa Barbara, CA), XEOMIN® (Merz Pharmaceuticals, GmbH., Frankfurt, Germany), NEURONOX® (Medy-Tox, Inc., Ochang-myeon, South Korea), BTX-A.

[091] As used herein, the term "non-naturally occurring BoNT/A" refers to any BoNT/A whose structure was modified with the aid of human manipulation, including, without limitation, a BoNT/A with an altered amino acid sequence produced by genetic engineering using random mutagenesis or rational design and a BoNT/A produced by in vitro chemical synthesis. Non-limiting examples of non-naturally occurring BoNT/As are described in, *e.g.*, Steward, L.E. et al., *Post-translational Modifications and Clostridial Neurotoxins*, U.S. Patent 7,223,577; Dolly, J.O. et al., *Activatable Clostridial Toxins*, U.S. Patent No. 7,419,676; Steward, L.E. et al., *Clostridial Neurotoxin Compositions and Modified Clostridial Neurotoxins*, US 2004/0220386; Steward, L.E. et al., *Modified Clostridial Toxins With Enhanced Targeting Capabilities For Endogenous Clostridial Toxin Receptor Systems*, U.S. Patent Publication No. 2008/0096248; Steward, L.E. et al., *Modified Clostridial Toxins With Altered Targeting Capabilities For Clostridial Toxin Target Cells*, U.S. Patent Publication No. 2008/0161543; Steward, L.E. et al., *Modified Clostridial Toxins With Enhanced Translocation Capabilities and Altered Targeting Activity For Clostridial Toxin Target Cells*, U.S. Patent Publication No. 2008/0241881, each of which is hereby incorporated by reference in its entirety.

[092] Thus in an embodiment, the BoNT/A activity being detected is from a naturally occurring BoNT/A. In aspects of this embodiment, the BoNT/A activity being detected is from a BoNT/A isoform or a BoNT/A subtype. In aspects of this embodiment, the BoNT/A activity being detected is from the BoNT/A of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In other aspects of this embodiment, the BoNT/A activity being detected is from a BoNT/A having, e.g., at least 70%, at least 75%, at least 80%, at

least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In other aspects of this embodiment, the BoNT/A activity being detected is from BOTOX®, DYSPORT®/RELOXIN®, PURTOX®, XEOMIN®, NEURONOX®, or BTX-A.

[093] In another embodiment, the BoNT/A activity being detected is from a non-naturally occurring BoNT/A. In other aspects of this embodiment, the BoNT/A activity being detected is from a non-naturally occurring BoNT/A variant having, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In other aspects of this embodiment, the BoNT/A activity being detected is from a non-naturally occurring BoNT/A variant having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more non-contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In yet other aspects of this embodiment, the BoNT/A activity being detected is from a non-naturally occurring BoNT/A variant having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

[094] Aspects of the present disclosure comprise, in part, a SNAP-25. As used herein, the term "SNAP-25" refers to a naturally-occurring SNAP-25 or a non-naturally occurring SNAP-25 which is preferentially cleaved by a BoNT/A. As used herein, the term "preferentially cleaved" refers to that the cleavage rate of BoNT/A substrate by a BoNT/A is at least one order of magnitude higher than the cleavage rate of any other substrate by BoNT/A. In aspects of this embodiment, the cleavage rate of BoNT/A substrate by a BoNT/A is at least two orders of magnitude higher, at least three orders of magnitude higher, at least four orders of magnitude higher, or at least five orders of magnitude higher then that the cleavage rate of any other substrate by BoNT/A.

[095] As used herein, the term "naturally occurring SNAP-25" refers to any SNAP-25 produced by a naturally-occurring process, including, without limitation, SNAP-25 isoforms produced from a post-translational modification, an alternatively-spliced transcript, or a spontaneous mutation, and SNAP-25 subtypes. A naturally occurring SNAP-25 includes, without limitation, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24, or one that substitutes, deletes or adds, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO:

16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[096] As used herein, the term "non-naturally occurring SNAP-25" refers to any SNAP-25 whose structure was modified with the aid of human manipulation, including, without limitation, a SNAP-25 produced by genetic engineering using random mutagenesis or rational design and a SNAP-25 produced by in vitro chemical synthesis. Non-limiting examples of non-naturally occurring SNAP-25s are described in, *e.g.*, Steward, L.E. et al., *FRET Protease Assays for Clostridial Toxins*, U.S. Patent 7,332,567; Fernandez-Salas et al., *Lipohilic Dye-based FRET Assays for Clostridial Toxin Activity*, U.S. Patent Publication 2008/0160561, each of which is hereby incorporated by reference in its entirety. A non-naturally occurring SNAP-25 may substitute, delete or add, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[097] Thus in an embodiment, a SNAP-25 is a naturally occurring SNAP-25. In aspects of this embodiment, the SNAP-25 is a SNAP-25 isoform or a SNAP-25 subtype. In aspects of this embodiment, the naturally occurring SNAP-25 is the naturally occurring SNAP-25 of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In other aspects of this embodiment, the SNAP-25 is a naturally occurring SNAP-25 having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[098] In another embodiment, a SNAP-25 is a non-naturally occurring SNAP-25. In other aspects of this embodiment, the SNAP-25 is a non-naturally occurring SNAP-25 having, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. In other aspects of this embodiment, the SNAP-25 is a non-naturally occurring SNAP-25 having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more non-contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO:

18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In yet other aspects of this embodiment, the SNAP-25 is a non-naturally occurring SNAP-25 having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[099] A SNAP-25 can be an endogenous SNAP-25 or an exogenous SNAP-25. As used herein, the term "endogenous SNAP-25" refers to a SNAP-25 naturally present in the cell because it is naturally encoded within the cell's genome, such that the cell inherently expresses the SNAP-25 without the need an external source of SNAP-25 or an external source of genetic material encoding a SNAP-25. The expression of an endogenous SNAP-25 may be with or without environmental stimulation such as, *e.g.*, cell differentiation. By definition, an endogenous SNAP-25 can only be a naturally-occurring SNAP-25 or variants thereof. For example, the following established cell lines express an endogenous SNAP-25: BE(2)-M17, Kelly, LA1-55n, N1E-115, N4TG3, N18, Neuro-2a, NG108-15, PC12, SH-SY5Y, SiMa, and SK-N-BE(2)-C.

[0100] As used herein, the term "exogenous SNAP-25" refers to a SNAP-25 expressed in a cell through the introduction of an external source of SNAP-25 or an external source of genetic material encoding a SNAP-25 by human manipulation. The expression of an exogenous SNAP-25 may be with or without environmental stimulation such as, *e.g.*, cell differentiation. As a non-limiting example, cells from an established cell line can express an exogenous SNAP-25 by transient or stably transfection of a SNAP-25. As another non-limiting example, cells from an established cell line can express an exogenous SNAP-25 by protein transfection of a SNAP-25. An exogenous SNAP-25 can be a naturally-occurring SNAP-25 or variants thereof, or a non-naturally occurring SNAP-25 or variants thereof.

[0101] Thus in an embodiment, cells from an established cell line express an endogenous SNAP-25. In aspects of this embodiment, the endogenous SNAP-25 expressed by cells from an established cell line is a naturally-occurring SNAP-25. In other aspects of this embodiment, the endogenous SNAP-25 expressed by cells from an established cell line is SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In yet aspects of this embodiment, the endogenous SNAP-25 expressed by cells from an established cell line is a naturally occurring SNAP-25, such as, *e.g.*, a SNAP-25 isoform or a SNAP-25 subtype. In other aspects of this embodiment, the endogenous SNAP-25 expressed by cells from an established cell line is a naturally occurring SNAP-25 having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at

least 95% amino acid identity with SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0102] In another embodiment, cells from an established cell line are transiently or stably engineered to express an exogenous SNAP-25. In an aspect of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally-occurring SNAP-25. In other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express the naturally-occurring SNAP-25 of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In yet other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally occurring SNAP-25, such as, *e.g.*, a SNAP-25 isoform or a SNAP-25 subtype. In still other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally occurring SNAP-25 having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0103] In another aspect of the embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring SNAP-25. In other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring SNAP-25 having, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring SNAP-25 having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more non-contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24. In yet other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring SNAP-25 having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or

more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0104] Assays that detect the cleavage of a BoNT/A substrate after exposure to a BoNT/A can be used to assess whether a cell is expressing an endogenous or exogenous SNAP-25. In these assays, generation of a SNAP-25 cleavage-product would be detected in cells expressing a SNAP-25 after BoNT/A treatment. Non-limiting examples of specific Western blot analysis, as well as well-characterized reagents, conditions and protocols are readily available from commercial vendors that include, without limitation, Amersham Biosciences, Piscataway, NJ; Bio-Rad Laboratories, Hercules, CA; Pierce Biotechnology, Inc., Rockford, IL; Promega Corporation, Madison, WI, and Stratagene, Inc., La Jolla, CA. It is understood that these and similar assays for SNAP-25 cleavage can be useful in identifying cells expressing an endogenous or an exogenous SNAP-25.

[0105] As non-limiting examples, Western blot analysis using an antibody that recognizes BoNT/A SNAP-25-cleaved product or both the cleaved and uncleaved forms of SNAP-25 can be used to assay for uptake of BoNT/A. Examples of α-SNAP-25 antibodies useful for these assays include, without limitation, α-SNAP-25 mouse monoclonal antibody SMI-81 (Sternberger Monoclonals Inc., Lutherville, MD), mouse α-SNAP-25 monoclonal antibody Cl 71.1 (Synaptic Systems, Goettingen, Germany), α-SNAP-25 mouse monoclonal antibody Cl 71.2 (Synaptic Systems, Goettingen, Germany), α-SNAP-25 mouse monoclonal antibody SP12 (Abcam, Cambridge, MA), α-SNAP-25 rabbit polyclonal antiserum (Synaptic Systems, Goettingen, Germany), α-SNAP-25 rabbit polyclonal antiserum (Abcam, Cambridge, MA), and α-SNAP-25 rabbit polyclonal antiserum S9684 (Sigma, St Louis, MO).

[0106] Aspects of the present disclosure comprise, in part, a BoNT/A receptor. As used herein, the term "BoNT/A receptor" refers to either a naturally-occurring BoNT/A receptor or a non-naturally occurring BoNT/A receptor which preferentially interacts with BoNT/A in a manner that elicits a BoNT/A intoxication response. As used herein, the term "preferentially interacts" refers to that the equilibrium dissociation constant (KD) of BoNT/A for a BoNT/A receptor is at least one order of magnitude less than that of BoNT/A for any other receptor at the cell surface. The equilibrium dissociation constant, a specific type of equilibrium constant that measures the propensity of an BoNT/A-BoNT/A receptor complex to separate (dissociate) reversibly into its component molecules, namely the BoNT/A and the BoNT/A receptor, is defined as KD=Ka/Kd at equilibrium. The association constant (Ka) is defined as Ka=[C]/[L][R] and the disassociation constant (Kd) is defined as Kd=[L][R]/[C], where [L] equals the molar concentration of BoNT/A, [R] is the molar concentration of a BoNT/A receptor, and [C] is the molar concentration of the BoNT/A-BoNT/A receptor complex, and where all concentrations are of such components when the system is at equilibrium. The smaller the dissociation constant, the more tightly bound the BoNT/A is to

its receptor, or the higher the binding affinity between BoNT/A and BoNT/A receptor. In aspects of this embodiment, the disassociation constant of BoNT/A for a BoNT/A receptor is at least two orders of magnitude less, at least three orders of magnitude less, at least four orders of magnitude less, or at least five orders of magnitude less than that of BoNT/A for any other receptor. In other aspects of this embodiment, the binding affinity of a BoNT/A that preferentially interacts with a BoNT/A receptor can have an equilibrium disassociation constant (KD) of, *e.g.*, of 500 nM or less, 400 nM or less, 300 nM or less, 200 nM, or less 100 nM or less. In other aspects of this embodiment, the binding affinity of a BoNT/A that preferentially interacts with a BoNT/A receptor can have an equilibrium disassociation constant (KD) of, *e.g.*, of 90 nM or less, 80 nM or less, 70 nM or less, 60 nM, 50 nM or less, 40 nM or less, 30 nM or less, 20 nM, or less 10 nM or less. As used herein, the term "elicits a BoNT/A intoxication response" refers to the ability of a BoNT/A receptor to interact with a BoNT/A to form a neurotoxin/receptor complex and the subsequent internalization of that complex into the cell cytoplasm.

[0107] As used herein, the term "naturally occurring BoNT/A receptor" refers to any BoNT/A receptor produced by a naturally-occurring process, including, without limitation, BoNT/A receptor isoforms produced from a post-translational modification, an alternatively-spliced transcript, or a spontaneous mutation, and BoNT/A receptor subtypes. A naturally occurring BoNT/A receptor includes, without limitation, a fibroblast growth factor receptor 2 (FGFR2), a fibroblast growth factor receptor 3 (FGFR3), a synaptic vesicle glycoprotein 2 (SV2), and a complex ganglioside like GT1b, such as those described in Ester Fernandez-Salas, et al., *Botulinum Toxin Screening Assays*, U.S. Patent Publication 2008/0003240; Ester Fernandez-Salas, et al., *Botulinum Toxin Screening Assays*, U.S. Patent Publication 2008/0182799; Min Dong et al., *SV2 is the Protein Receptor for Botulinum Neurotoxin A*, Science (2006); S. Mahrhold et al, *The Synaptic Vesicle Protein 2C Mediates the Uptake of Botulinum Neurotoxin A into Phrenic Nerves*, 580(8) FEBS Lett. 2011-2014 (2006), each of which is hereby incorporated by reference in its entirety. A naturally occurring FGFR2 includes, without limitation, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, and SEQ ID NO: 70, or one that substitutes, deletes or adds, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, and SEQ ID NO: 70. A naturally occurring FGFR3 includes, without limitation, SEQ ID NO: 25, SEQ ID NO: 26, and SEQ ID NO: 27, or one that substitutes, deletes or adds, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 25, SEQ ID NO: 26, and SEQ ID NO: 27. A naturally occurring SV2 includes, without limitation, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, and SEQ ID NO: 31, or one that substitutes, deletes or adds, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, and SEQ ID NO: 31.

[0108] As used herein, the term "non-naturally occurring BoNT/A receptor variant" refers to any BoNT/A receptor produced with the aid of human manipulation or design, including, without limitation, a BoNT/A receptor produced by genetic engineering using random mutagenesis or rational design and a BoNT/A receptor produced by chemical synthesis. Non-limiting examples of non-naturally occurring BoNT/A variants include, *e.g.*, conservative BoNT/A receptor variants, non-conservative BoNT/A receptor variants, BoNT/A receptor chimeric variants and active BoNT/A receptor fragments.

[0109] As used herein, the term "non-naturally occurring BoNT/A receptor" refers to any BoNT/A receptor whose structure was modified with the aid of human manipulation, including, without limitation, a BoNT/A receptor produced by genetic engineering using random mutagenesis or rational design and a BoNT/A receptor produced by in vitro chemical synthesis. Non-limiting examples of non-naturally occurring BoNT/A receptors are described in, *e.g.*, Ester Fernandez-Salas, et al., *Botulinum Toxin Screening Assays*, U.S. Patent Publication 2008/0003240; Ester Fernandez-Salas, et al., *Botulinum Toxin Screening Assays*, U.S. Patent Publication 2008/0182799, each of which is hereby incorporated by reference in its entirety. A non-naturally occurring BoNT/A receptor may substitute, delete or add, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more amino acids from SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70.

[0110] Thus in an embodiment, a BoNT/A receptor is a naturally occurring BoNT/A receptor such as, *e.g.*, FGFR2, FGFR3 or SV2. In aspects of this embodiment, the BoNT/A receptor is a BoNT/A receptor isoform or a BoNT/A receptor subtype. In aspects of this embodiment, the naturally occurring BoNT/A receptor is the naturally occurring BoNT/A receptor of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In other aspects of this embodiment, the BoNT/A receptor is a naturally occurring BoNT/A receptor having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70.

[0111] In another embodiment, a BoNT/A receptor is a non-naturally occurring BoNT/A receptor, such as, *e.g.*, a genetically-engineered FGFR2, a genetically-engineered FGFR3, or a genetically-engineered SV2. In other aspects of this embodiment, the BoNT/A receptor is a non-naturally occurring BoNT/A receptor having, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%

amino acid identity with SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In other aspects of this embodiment, the BoNT/A receptor is a non-naturally occurring BoNT/A receptor having, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more non-contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In yet other aspects of this embodiment, the BoNT/A receptor is a non-naturally occurring BoNT/A receptor having, *e.g.*, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70.

[0112] A BoNT/A receptor can be an endogenous BoNT/A receptor or an exogenous BoNT/A receptor. As used herein, the term "endogenous BoNT/A receptor" refers to a BoNT/A receptor naturally present in the cell because it is naturally encoded within the cell's genome, such that the cell inherently expresses the BoNT/A receptor without the need an external source of BoNT/A receptor or an external source of genetic material encoding a BoNT/A receptor. Expression of an endogenous BoNT/A receptor may be with or without environmental stimulation such as *e.g.*, cell differentiation or promoter activation. For example, the following established cell lines express at least one endogenous BoNT/A receptor: BE(2)-M17, Kelly, LA1-55n, N1E-115, N4TG3, N18, Neuro-2a, NG108-15, PC12, SH-SY5Y, SiMa, and SK-N-BE(2)-C. An endogenous BoNT/A receptor can only be a naturally-occurring BoNT/A receptor or naturally-occurring variants thereof.

[0113] As used herein, the term "exogenous BoNT/A receptor" refers to a BoNT/A receptor expressed in a cell through the introduction of an external source of BoNT/A receptor or an external source of genetic material encoding a BoNT/A receptor by human manipulation. The expression of an exogenous BoNT/A receptor may be with or without environmental stimulation such as, *e.g.*, cell differentiation or promoter activation. As a non-limiting example, cells from an established cell line can express one or more exogenous BoNT/A receptors by transient or stably transfection of a polynucleotide molecule encoding a BoNT/A receptor, such as, *e.g.*, a FGFR2, a FGFR3, or a SV2. As another non-limiting example, cells from an established cell line can express one or more exogenous BoNT/A receptors by protein transfection of the BoNT/A receptors, such as, *e.g.*, a FGFR2, a FGFR3, or a SV2. An exogenous

BoNT/A receptor can be a naturally-occurring BoNT/A receptor or naturally occurring variants thereof, or non-naturally occurring BoNT/A receptor or non-naturally occurring variants thereof.

[0114] Thus in an embodiment, cells from an established cell line express an endogenous BoNT/A receptor. In aspects of this embodiment, the endogenous BoNT/A receptor expressed by cells from an established cell line is a naturally-occurring BoNT/A receptor. In other aspects of this embodiment, the endogenous BoNT/A receptor expressed by cells from an established cell line is SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In yet aspects of this embodiment, the endogenous BoNT/A receptor expressed by cells from an established cell line is a naturally occurring BoNT/A receptor, such as, *e.g.*, a BoNT/A receptor isoform or a BoNT/A receptor subtype. In other aspects of this embodiment, the endogenous BoNT/A receptor expressed by cells from an established cell line is a naturally occurring BoNT/A receptor having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70.

[0115] In another embodiment, cells from an established cell line are transiently or stably engineered to express an exogenous BoNT/A receptor. In an aspect of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally-occurring BoNT/A receptor. In other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express the naturally-occurring BoNT/A receptor of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In yet other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally occurring BoNT/A receptor, such as, *e.g.*, a BoNT/A receptor isoform or a BoNT/A receptor subtype. In still other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally occurring BoNT/A receptor having, e.g., at least 70% amino acid identity, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70.

[0116] In another aspect of the embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring BoNT/A receptor. In other aspects of this embodiment,

cells from an established cell line are transiently or stably engineered to express a non-naturally occurring BoNT/A receptor having, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% amino acid identity with SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring BoNT/A receptor having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more non-contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70. In yet other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally occurring BoNT/A receptor having, e.g., 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more contiguous amino acid substitutions, deletions, or additions relative to SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, or SEQ ID NO: 70

[0117] In another embodiment, cells from an established cell line are transiently or stably engineered to express an exogenous FGFR2, an exogenous FGFR3, an exogenous SV2, or any combination thereof. In aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a naturally-occurring FGFR2, a naturally-occurring FGFR3, a naturally-occurring SV2, or any combination thereof. In yet other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express a non-naturally-occurring FGFR2, a non-naturally-occurring FGFR3, a non-naturally-occurring SV2, or any combination thereof. In still other aspects of this embodiment, cells from an established cell line are transiently or stably engineered to express either a naturally-occurring FGFR2 or a non-naturally-occurring FGFR2, a naturally-occurring FGFR3 or a non-naturally-occurring FGFR3, a naturally-occurring SV2 or a non-naturally-occurring SV2, or any combination thereof.

[0118] Cells that express one or more endogenous or exogenous BoNT/A receptors can be identified by routine methods including direct and indirect assays for toxin uptake. Assays that determine BoNT/A binding or uptake properties can be used to assess whether a cell is expressing a BoNT/A receptor. Such assays include, without limitation, cross-linking assays using labeled BoNT/A, such as, e.g., [125I] BoNT/A, [125I], see, e.g., Noriko Yokosawa et al., *Binding of Clostridium botulinum type C neurotoxin to different neuroblastoma cell lines*, 57(1) Infect. Immun. 272-277 (1989); Noriko Yokosawa et al., *Binding*

of botulinum type CI, D and E neurotoxins to neuronal cell lines and synaptosomes, 29(2) Toxicon 261-264 (1991); and Tei-ichi Nishiki et al., *Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes*, 269(14) J. Biol. Chem. 10498-10503 (1994). Other non-limiting assays include immunocytochemical assays that detect BoNT/A binding using labeled or unlabeled antibodies, see, e.g., Atsushi Nishikawa et al., *The receptor and transporter for internalization of Clostridium botulinum type C progenitor toxin into HT-29 cells*, 319(2) Biochem. Biophys. Res. Commun. 327-333 (2004) and immunoprecipitation assays, see, e.g., Yukako Fujinaga et al., *Molecular characterization of binding subcomponents of Clostridium botulinum type C progenitor toxin for intestinal epithelial cells and erythrocytes*, 150(Pt 5) Microbiology 1529-1538 (2004), that detect bound toxin using labeled or unlabeled antibodies. Antibodies useful for these assays include, without limitation, antibodies selected against BoNT/A, antibodies selected against a BoNT/A receptor, such as, e.g., FGFR2, FGFR3, or SV2, and/or antibodies selected against a ganglioside, such as, e.g., GD1a, GD1b, GD3, GQ1b, or GT1b. If the antibody is labeled, the binding of the molecule can be detected by various means, including Western blot analysis, direct microscopic observation of the cellular location of the antibody, measurement of cell or substrate-bound antibody following a wash step, flow cytometry, electrophoresis or capillary electrophoresis, employing techniques well-known to those of skill in the art. If the antibody is unlabeled, one may employ a labeled secondary antibody for indirect detection of the bound molecule, and detection can proceed as for a labeled antibody. It is understood that these and similar assays that determine BoNT/A uptake properties or characteristics can be useful in identifying cells expressing endogenous or exogenous or BoNT/A receptors.

[0119] Assays that monitor the release of a molecule after exposure to BoNT/A can also be used to assess whether a cell is expressing one or more endogenous or exogenous BoNT/A receptors. In these assays, inhibition of the molecule's release would occur in cells expressing a BoNT/A receptor after BoNT/A treatment. Well known assays include methods that measure inhibition of radio-labeled catecholamine release from neurons, such as, e.g., [3H] noradrenaline or [3H] dopamine release, see e.g., A Fassio et al., *Evidence for calcium-dependent vesicular transmitter release insensitive to tetanus toxin and botulinum toxin type F*, 90(3) Neuroscience 893-902 (1999); and Sara Stigliani et al., *The sensitivity of catecholamine release to botulinum toxin C1 and E suggests selective targeting of vesicles set into the readily releasable pool*, 85(2) J. Neurochem. 409-421 (2003), or measures catecholamine release using a fluorometric procedure, see, e.g., Anton de Paiva et al., *A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly*, 268(28) J. Biol. Chem. 20838-20844 (1993); Gary W. Lawrence et al., *Distinct exocytotic responses of intact and permeabilised chromaffin cells after cleavage of the 25-kDa synaptosomal-associated protein (SNAP-25) or synaptobrevin by botulinum toxin A or B*, 236(3) Eur. J. Biochem. 877-886 (1996); and Patrick Foran et al., *Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release*, 35(8) Biochemistry 2630-2636 (1996). Other non-limiting examples include assays that measure inhibition of hormone release from endocrine cells,

such as, e.g., anterior pituitary cells or ovarian cells. It is understood that these and similar assays for molecule release can be useful in identifying cells expressing endogenous or exogenous or BoNT/A receptors.

[0120] Assays that detect the cleavage of a BoNT/A substrate after exposure to a BoNT/A can also be used to assess whether a cell is expressing one or more endogenous or exogenous BoNT/A receptors. In these assays, generation of a BoNT/A substrate cleavage-product, or disappearance of the intact BoNT/A substrate, would be detected in cells expressing a BoNT/A receptor after BoNT/A treatment. Non-limiting examples of specific Western blot analysis, as well as well-characterized reagents, conditions and protocols are readily available from commercial vendors that include, without limitation, Amersham Biosciences, Piscataway, NJ; Bio-Rad Laboratories, Hercules, CA; Pierce Biotechnology, Inc., Rockford, IL; Promega Corporation, Madison, WI, and Stratagene, Inc., La Jolla, CA. It is understood that these and similar assays for BoNT/A substrate cleavage can be useful in identifying cells expressing endogenous or exogenous BoNT/A receptors.

[0121] As non-limiting examples, Western blot analysis using an antibody that recognizes BoNT/A SNAP-25-cleaved product or both the cleaved and uncleaved forms of SNAP-25 can be used to assay for uptake of BoNT/A. Examples of α-SNAP-25 antibodies useful for these assays include, without limitation, SMI-81 α-SNAP-25 mouse monoclonal antibody (Sternberger Monoclonals Inc., Lutherville, MD), CI 71.1 mouse α-SNAP-25 monoclonal antibody (Synaptic Systems, Goettingen, Germany), CI 71.2 α-SNAP-25 mouse monoclonal antibody (Synaptic Systems, Goettingen, Germany), SP12 α-SNAP-25 mouse monoclonal antibody (Abcam, Cambridge, MA), α-SNAP-25 rabbit polyclonal antiserum (Synaptic Systems, Goettingen, Germany), α-SNAP-25 rabbit polyclonal antiserum S9684 (Sigma, St. Louis, MO), and α-SNAP-25 rabbit polyclonal antiserum (Abcam, Cambridge, MA).

[0122] Aspects of the present disclosure provide cells that through genetic manipulation or recombinant engineering are made to expresses an exogenous SNAP-25 and/or one or more exogenous BoNT/A receptors. Cells useful to express an exogenous SNAP-25 and/or one or more exogenous BoNT/A receptors through genetic manipulation or recombinant engineering include neuronal cells and non-neuronal cells that may or may not express an endogenous SNAP-25 and/or one or more endogenous BoNT/A receptors. It is further understood that such genetically manipulated or recombinantly engineered cells may express an exogenous SNAP-25 and one or more exogenous BoNT/A receptors under control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both. It is understood that any cell is useful as long as the cell can be genetically manipulated or recombinantly engineered to expresses an exogenous SNAP-25 and/or one or more exogenous BoNT/A receptors and is capable of undergoing BoNT/A intoxication.

[0123] Methods useful for introducing into a cell an exogenous polynucleotide molecule encoding a component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A

proteolytically cleaves a SNAP-25 substrate, such as, *e.g.*, a SNAP-25, a FGFR2, a FGFR3, or a SV2, include, without limitation, chemical-mediated delivery methods, such as, *e.g.*, calcium phosphate-mediated, diethyl-aminoethyl (DEAE) dextran-mediated, lipid-mediated, polyethyleneimine (PEI)-mediated, polylysine-mediated and polybrene-mediated; physical-mediated delivery methods, such as, *e.g.*, biolistic particle delivery, microinjection, protoplast fusion and electroporation; and viral-mediated delivery methods, such as, *e.g.*, retroviral-mediated transfection, *see e.g.*, *Introducing Cloned Genes into Cultured Mammalian Cells*, pp. 16.1-16.62 (Sambrook & Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3rd ed. 2001); Alessia Colosimo et al., *Transfer and Expression of Foreign Genes in Mammalian Cells*, 29(2) Biotechniques 314-318, 320-322, 324 (2000); Philip Washbourne & A. Kimberley McAllister, *Techniques for Gene Transfer into Neurons*, 12(5) Curr. Opin. Neurobiol. 566-573 (2002); and Current Protocols in Molecular Biology, John Wiley and Sons, pp 9.16.4-9.16.11 (2000), each of which is incorporated by reference in its entirety. One skilled in the art understands that selection of a specific method to introduce a polynucleotide molecule into a cell will depend, in part, on whether the cell will transiently or stably contain a component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate. Non-limiting examples of polynucleotide molecule encoding a component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate as as follows: FGFR2 polynucleotide molecule of SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, or SEQ ID NO: 138; FGFR3 polynucleotide molecule of SEQ ID NO: 139, SEQ ID NO: 140, or SEQ ID NO: 141; SV2 polynucleotide molecule of SEQ ID NO: 142, SEQ ID NO: 143, or SEQ ID NO: 144; and SNAP-25 polynucleotide molecule of SEQ ID NO: 145, or SEQ ID NO: 146.

[0124] Chemical-mediated delivery methods are well-known to a person of ordinary skill in the art and are described in, *e.g.*, Martin Jordan & Florian Worm, *Transfection of Adherent and Suspended Cells by Calcium Phosphate*, 33(2) Methods 136-143 (2004); Chun Zhang et al., *Polyethylenimine Strategies for Plasmid Delivery to Brain-Derived Cells*, 33(2) Methods 144-150 (2004), each of which is hereby incorporated by reference in its entirety. Such chemical-mediated delivery methods can be prepared by standard procedures and are commercially available, see, *e.g.*, CellPhect Transfection Kit (Amersham Biosciences, Piscataway, NJ); Mammalian Transfection Kit, Calcium phosphate and DEAE Dextran, (Stratagene, Inc., La Jolla, CA); Lipofectamine™ Transfection Reagent (Invitrogen, Inc., Carlsbad, CA); ExGen 500 Transfection kit (Fermentas, Inc., Hanover, MD), and SuperFect and Effectene Transfection Kits (Qiagen, Inc., Valencia, CA).

[0125] Physical-mediated delivery methods are well-known to a person of ordinary skill in the art and are described in, *e.g.*, Jeike E. Biewenga et al., *Plasmid-Mediated Gene Transfer in Neurons using the Biolistics Technique*, 71(1) J. Neurosci. Methods. 67-75 (1997); John O'Brien & Sarah C. R. Lummis, *Biolistic and Diolistic Transfection: Using the Gene Gun to Deliver DNA and Lipophilic Dyes into Mammalian Cells*, 33(2) Methods 121-125 (2004); M. Golzio et al., *In Vitro and In Vivo Electric Field-*

Mediated Permeabilization, Gene Transfer, and Expression, 33(2) Methods 126-135 (2004); and Oliver Greschet al., *New Non-Viral Method for Gene Transfer into Primary Cells*, 33(2) Methods 151-163 (2004), each of which is hereby incorporated by reference in its entirety.

[0126] Viral-mediated delivery methods are well-known to a person of ordinary skill in the art and are described in, *e.g.*, Chooi M. Lai et al., *Adenovirus and Adeno-Associated Virus Vectors*, 21(12) DNA Cell Biol. 895-913 (2002); Ilya Frolov et al., *Alphavirus-Based Expression Vectors: Strategies and Applications*, 93(21) Proc. Natl. Acad. Sci. U. S. A. 11371-11377 (1996); Roland Wolkowicz et al., *Lentiviral Vectors for the Delivery of DNA into Mammalian Cells*, 246 Methods Mol. Biol. 391-411 (2004); A. Huser & C. Hofmann, *Baculovirus Vectors: Novel Mammalian Cell Gene-Delivery Vehicles and Their Applications*, 3(1) Am. J. Pharmacogenomics 53-63 (2003); Tiziana Tonini et al., *Transient Production of Retroviral- and Lentiviral-Based Vectors for the Transduction of Mammalian Cells*, 285 Methods Mol. Biol. 141-148 (2004); Manfred Gossen & Hermann Bujard, *Tight Control of Gene Expression in Eukaryotic Cells by Tetracycline-Responsive Promoters*, U.S. Patent No. 5,464,758; Hermann Bujard & Manfred Gossen, *Methods for Regulating Gene Expression*, U.S. Patent No. 5,814,618; David S. Hogness, *Polynucleotides Encoding Insect Steroid Hormone Receptor Polypeptides and Cells Transformed With Same*, U.S. Patent No. 5,514,578; David S. Hogness, *Polynucleotide Encoding Insect Ecdysone Receptor*, U.S. Patent 6,245,531; Elisabetta Vegeto et al., *Progesterone Receptor Having C. Terminal Hormone Binding Domain Truncations*, U.S. Patent No. 5,364,791; Elisabetta Vegeto et al., *Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy*, U.S. Patent No. 5,874,534, each of which is hereby incorporated by reference in its entirety. Such viral-mediated delivery methods can be prepared by standard procedures and are commercially available, see, e.g., ViraPower™ Adenoviral Expression System (Invitrogen, Inc., Carlsbad, CA) and ViraPower™ Adenoviral Expression System Instruction Manual 25-0543 version A, Invitrogen, Inc., (Jul. 15, 2002); and AdEasy™ Adenoviral Vector System (Stratagene, Inc., La Jolla, CA) and AdEasy™ Adenoviral Vector System Instruction Manual 064004f, Stratagene, Inc. Furthermore, such viral delivery systems can be prepared by standard methods and are commercially available, see, e.g., BD™ Tet-Off and Tet-On Gene Expression Systems (BD Biosciences-Clonetech, Palo Alto, CA) and BD™ Tet-Off and Tet-On Gene Expression Systems User Manual, PT3001-1, BD Biosciences Clonetech, (Mar. 14, 2003), GeneSwitch™ System (Invitrogen, Inc., Carlsbad, CA) and GeneSwitch™ System A Mifepristone-Regulated Expression System for Mammalian Cells version D, 25-0313, Invitrogen, Inc., (Nov. 4, 2002); ViraPower™ Lentiviral Expression System (Invitrogen, Inc., Carlsbad, CA) and ViraPower™ Lentiviral Expression System Instruction Manual 25-0501 version E, Invitrogen, Inc., (Dec. 8, 2003); and Complete Control® Retroviral Inducible Mammalian Expression System (Stratagene, La Jolla, CA) and Complete Control® Retroviral Inducible Mammalian Expression System Instruction Manual, 064005e.

[0127] Thus, in an embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain a polynucleotide molecule encoding a component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate. In

another embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain a polynucleotide molecule encoding a plurality of components necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate. In aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain a polynucleotide molecule encoding FGFR2, FGFR3, SV2 or SNAP-25. In aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain the polynucleotide molecule encoding FGFR2 of SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, or SEQ ID NO: 138. In other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain the polynucleotide molecule encoding FGFR3 of SEQ ID NO: 139, SEQ ID NO: 140, or SEQ ID NO: 141. In yet other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain the polynucleotide molecule encoding SV2 of SEQ ID NO: 142, SEQ ID NO: 143, or SEQ ID NO: 144. In yet other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication transiently contain the polynucleotide molecule encoding SNAP-25 of SEQ ID NO: 145, or SEQ ID NO: 146.

[0128] In another embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain a polynucleotide molecule encoding a component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate. In another embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain a polynucleotide molecule encoding a plurality of components necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate. In aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain a polynucleotide molecule encoding FGFR2, FGFR3, SV2 or SNAP-25. In aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain the polynucleotide molecule encoding FGFR2 of SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, or SEQ ID NO: 138. In other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain the polynucleotide molecule encoding FGFR3 of SEQ ID NO: 139, SEQ ID NO: 140, or SEQ ID NO: 141. In yet other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain the polynucleotide molecule encoding SV2 of SEQ ID NO: 142, SEQ ID NO: 143, or SEQ ID NO: 144. In yet other aspects of this embodiment, cells from an established cell line susceptible to BoNT/A intoxication stably contain the polynucleotide molecule encoding SNAP-25 of SEQ ID NO: 145, or SEQ ID NO: 146.

[0129] As mentioned above, an exogenous component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate, such as, *e.g.*, a SNAP-25, a FGFR2, a FGFR3, or a SV2 disclosed in the present specification can be introduced into a cell. Any and all methods useful for introducing such an exogenous component with a delivery agent into

a cell population can be useful with the proviso that this method transiently introduces the exogenous component disclosed in the present specification in at least 50% of the cells within a given cell population. Thus, aspects of this embodiment can include a cell population in which, e.g., at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the given cell population transiently contains an exogenous component necessary for the cells to undergo the overall cellular mechanism whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate, such as, *e.g.*, a SNAP-25, a FGFR2, a FGFR3, or a SV2 disclosed in the present specification. As used herein, the term "delivery agent" refers to any molecule that enables or enhances internalization of a covalently-linked, non-covalently-linked or in any other manner associated with a polypeptide into a cell. Thus, the term "delivery agent" encompasses, without limitation, proteins, peptides, peptidomimetics, small molecules, polynucleotide molecules, liposomes, lipids, viruses, retroviruses and cells that, without limitation, transport a covalently or non-covalently linked molecule to the cell membrane, cell cytoplasm or nucleus. It further is understood that the term "delivery agent" encompasses molecules that are internalized by any mechanism, including delivery agents which function via receptor mediated endocytosis and those which are independent of receptor mediated endocytosis.

[0130] A delivery agent can also be an agent that enables or enhances cellular uptake of a covalently linked component, like FGFR2, FGFR3, SV2, or SNAP-25, such as, *e.g.*, by chemical conjugation or by genetically produced fusion proteins. Methods that covalently link delivery agents and methods of using such agents are described in, e.g., Steven F. Dowdy, *Protein Transduction System and Methods of Use Thereof*, International Publication No WO 00/34308; Gérard Chassaing & Alain Prochiantz, *Peptides which can be Used as Vectors for the Intracellular Addressing of Active Molecules*, U.S. Patent No. 6,080,724; Alan Frankel et al., *Fusion Protein Comprising TAT-derived Transport Moiert*, U.S. Patent No. 5,674,980; Alan Frankel et al., *TAT-derived Transport Polypeptide Conjugates*, U.S. Patent No. 5,747,641; Alan Frankel et al., *TAT-derived Transport Polypeptides and Fusion Proteins*, U.S. Patent No. 5,804,604; Peter F. J. O'Hare et al., *Use of Transport Proteins*, U.S. Patent No. 6,734,167; Yao-Zhong Lin & Jack J. Hawiger, *Method for Importing Biologically Active Molecules into Cells*, U.S. Patent No. 5,807,746; Yao-Zhong Lin & Jack J. Hawiger, *Method for Importing Biologically Active Molecules into Cells*, U.S. Patent No. 6,043,339; Yao-Zhong Lin et al., *Sequence and Method for Genetic Engineering of Proteins with Cell Membrane Translocating Activity*, U.S. Patent No. 6,248,558; Yao-Zhong Lin et al., Sequence and Method for Genetic *Engineering of Proteins with Cell Membrane Translocating Activity*, U.S. Patent No. 6,432,680; Jack J. Hawiger et al., *Method for Importing Biologically Active Molecules into Cells*, U.S. Patent No. 6,495,518; Yao-Zhong Lin et al., *Sequence and Method for Genetic Engineering of Proteins with Cell Membrane Translocating Activity*, U.S. Patent No. 6,780,843; Jonathan B. Rothbard & Paul A Wender, *Method and Composition for Enhancing Transport Across Biological Membranes*, U.S. Patent No. 6,306,993; Jonathan B. Rothbard & Paul A Wender, *Method and Composition for Enhancing Transport Across Biological Membranes*, U.S. Patent No. 6,495,663; and Pamela B. Davis et al., *Fusion Proteins for Protein Delivery*, U.S. Patent No. 6,287,817, each of which is incorporated by reference in its entirety.

[0131] A delivery agent can also be an agent that enables or enhances cellular uptake of a non-covalently associated component, like FGFR2, FGFR3, SV2c, or SNAP-25. Methods that function in the absence of covalent linkage and methods of using such agents are described in, e.g., Gilles Divita et al, *Peptide-Mediated Transfection Agents and Methods of Use*, U.S. Patent No. 6,841,535; Philip L Felgner and Olivier Zelphati, *Intracellular Protein Delivery Compositions and Methods of Use*, U.S. Patent Publication No. 2003/0008813; and Michael Karas, *Intracellular Delivery of Small Molecules, Proteins and Nucleic Acids*, U.S. Patent Publication 2004/0209797, each of which is incorporated by reference in its entirety. Such peptide delivery agents can be prepared and used by standard methods and are commercially available, see, e.g. the CHARIOT™ Reagent (Active Motif, Carlsbad, CA); BIO-PORTER® Reagent (Gene Therapy Systems, Inc., San Diego, CA), BIO TREK™ Protein Delivery Reagent (Stratagene, La Jolla, CA), and PRO-JECT™ Protein Transfection Reagent (Pierce Biotechnology Inc., Rockford, IL).

[0132] Aspects of the present disclosure comprise, in part, a sample comprising a BoNT/A. As used herein, the term "sample comprising a BoNT/A" refers to any biological matter that contains or potentially contains an active BoNT/A. A variety of samples can be assayed according to a method disclosed in the present specification including, without limitation, purified, partially purified, or unpurified BoNT/A; recombinant single chain or di-chain toxin with a naturally or non-naturally occurring sequence; recombinant BoNT/A with a modified protease specificity; recombinant BoNT/A with an altered cell specificity; bulk BoNT/A; a formulated BoNT/A product, including, *e.g.*, BOTOX®, DYSPORT®/RELOXIN®, XEOMIN®, PURTOX®, NEURONOX®, BTX-A and; cells or crude, fractionated or partially purified cell lysates from, *e.g.*, bacteria, yeast, insect, or mammalian sources; blood, plasma or serum; raw, partially cooked, cooked, or processed foods; beverages; animal feed; soil samples; water samples; pond sediments; lotions; cosmetics; and clinical formulations. It is understood that the term sample encompasses tissue samples, including, without limitation, mammalian tissue samples, livestock tissue samples such as sheep, cow and pig tissue samples; primate tissue samples; and human tissue samples. Such samples encompass, without limitation, intestinal samples such as infant intestinal samples, and tissue samples obtained from a wound. As non-limiting examples, a method of detecting picomolar amounts of BoNT/A activity can be useful for determining the presence or activity of a BoNT/A in a food or beverage sample; to assay a sample from a human or animal, for example, exposed to a BoNT/A or having one or more symptoms of botulism; to follow activity during production and purification of bulk BoNT/A; to assay a formulated BoNT/A product used in pharmaceutical or cosmetics applications; or to assay a subject's blood serum for the presence or absence of neutralizing α-BoNT/A antibodies.

[0133] Thus, in an embodiment, a sample comprising a BoNT/A is a sample comprising any amount of a BoNT/A. In aspects of this embodiment, a sample comprising a BoNT/A comprises about 100 ng or less, about 10 ng or less, about 1 ng or less, about 100 pg or less, about 10 pg or less, or about 1 pg or less of a BoNT/A. In other aspects of this embodiment, a sample comprising a BoNT/A comprises about 1 μM or

less, about 100 nM or less, about 10 nM or less, about 1 nM or less, about 100 pM or less, about 10 pM or less, about 1 pM or less, about 100 fM or less, about 10 fM or less, or about 1 fM or less of a BoNT/A.

[0134] Aspects of the present disclosure comprise, in part, isolating from the treated cell a SNAP-25 component comprising a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. As used herein, the term "SNAP-25 component comprising a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond" refers to a cellular component containing the SNAP-25 cleavage product. It is envisioned that any method suitable for enriching or isolating a SNAP-25 component can be useful, including, without limitation, cell lysing protocols, spin-column purification protocols, immunoprecipitation, affinity purification, and protein chromatography.

[0135] Aspects of the present disclosure comprise, in part, an α-SNAP-25 antibody linked to a solid phase support. As used herein, the term "solid-phase support" is synonymous with "solid phase" and refers to any matrix that can be used for immobilizing an α-SNAP-25 antibody disclosed in the present specification. Non-limiting examples of solid phase supports include, e.g., a tube; a plate; a column; pins or "dipsticks"; a magnetic particle, a bead or other spherical or fibrous chromatographic media, such as, e.g., agarose, sepharose, silica and plastic; and sheets or membranes, such as, e.g., nitrocellulose and polyvinylidene fluoride (PVDF). The solid phase support can be constructed using a wide variety of materials such as, *e.g.*, glass, carbon, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, nylon, diazocellulose, or starch. The solid phase support selected can have a physical property that renders it readily separable from soluble or unbound material and generally allows unbound materials, such as, *e.g.*, excess reagents, reaction by-products, or solvents, to be separated or otherwise removed (by, *e.g.*, washing, filtration, centrifugation, etc.) from solid phase support-bound assay component. Non-limiting examples of how to make and use a solid phase supports are described in, e.g., Molecular Cloning, A Laboratory Manual, *supra*, (2001); and Current Protocols in Molecular Biology, *supra*, (2004), each of which is hereby incorporated by reference in its entirety.

[0136] Aspects of the present disclosure comprise, in part, detecting the presence of an antibody-antigen complex comprising an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. It is envisioned that any detection system can be used to practice aspects of this disclosed immuno-based method, with the provision that the signal to noise ratio can distinguish to a statistically significant degree the signal from the antibody-antigen complex from the background signal. Non-limiting examples of immuno-based detection systems include immunoblot analysis, like Western blotting and dot-blotting, immunoprecipitation analysis, enzyme-linked immunosorbent analysis (ELISA), and sandwich ELISA. The detection of the signal can be achieved using autoradiography with imaging or phosphorimaging (AU), chemiluminescense (CL), electrochemiluminescence (ECL), bioluminescence (BL), fluorescence,

resonance energy transfer, plane polarization, colormetric, or flow cytometry (FC). Descriptions of immuno-based detection systems are disclosed in, *e.g.*, Michael M.Rauhut, Chemiluminescence, In Kirk-Othmer Concise Encyclopedia of Chemical Technology (Ed. Grayson, 3rd ed, John Wiley and Sons, 1985); A. W. Knight, *A Review of Recent Trends in Analytical Applications of Electrogenerated Chemiluminescence*, Trends Anal. Chem. 18(1): 47-62 (1999); K. A. Fahnrich, et al., *Recent Applications of Electrogenerated Chemiluminescence in Chemical Analysis*, Talanta 54(4): 531-559 (2001); *Commonly Used Techniques in Molecular Cloning*, pp. A8.1-A8-55 (Sambrook & Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3rd ed. 2001); *Detection Systems*, pp. A9.1-A9-49 (Sambrook & Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3rd ed. 2001); Electrogenerated Chemiluminescence, (Ed. Allen J. Bard, Marcel Dekker, Inc., 2004), each of which is hereby incorporated by reference in its entirety.

[0137] A sandwich ELISA (or sandwich immunoassay) is a method based on two antibodies, which bind to different epitopes on the antigen. A capture antibody having a high binding specificity for the antigen of interest, is bound to a solid surface. The antigen is then added followed by addition of a second antibody referred to as the detection antibody. The detection antibody binds the antigen to a different epitope than the capture antibody. The antigen is therefore 'sandwiched' between the two antibodies. The antibody binding affinity for the antigen is usually the main determinant of immunoassay sensitivity. As the antigen concentration increases the amount of detection antibody increases leading to a higher measured response. To quantify the extent of binding different reporter systems can be used, such as, *e.g.*, an enzyme attached to the secondary antibody and a reporter substrate where the enzymatic reaction forms a readout as the detection signal. The signal generated is proportional to the amount of target antigen present in the sample. The reporter substrate used to measure the binding event determines the detection mode. A spectrophotometric plate reader is used for colorimetric detection. Chemiluminescent and electrochemiluminescence substrates have been developed which further amplify the signal and can be read on a luminescent reader. The reporter can also be a fluorescent readout where the enzyme step of the assay is replaced with a fluorophore and the readout is then measured using a fluorescent reader. Reagents and protocols necessary to perform an ECL sandwich ELISA are commercially available, including, without exception, MSD sandwich ELISA-ECL detection platform (Meso Scale Discovery, Gaithersburg, MD).

[0138] Thus, in an embodiment, detecting the presence of an antibody-antigen complex comprising an α-SNAP-25 antibody that selectively binds to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond can be performed using an immuno-blot analysis, an immunoprecipitation analysis, an ELISA, or a sandwich ELISA. In aspects of this embodiment, the detection is performed using a AU, CL, ECL, or BL immuno-blot analysis, a AU, CL, ECL, BL, or FC immunoprecipitation analysis, a AU, CL, ECL, BL, or FC ELISA, or a AU, CL, ECL, BL, or FC sandwich ELISA.

[0139] Aspects of the present disclosure can be practiced in a singleplex or multiplex fashion. An immuno-based method of detecting BoNT/A activity practiced in a single-plex fashion is one that only detects the presence of an antibody-antigen complex comprising an α-SNAP-25 antibody and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. An immuno-based method of detecting BoNT/A activity practiced in a multiplex fashion is one that concurrently detects the presence of two or more antibody-antigen complexes; one of which is the antibody-antigen complex comprising an α-SNAP-25 antibody and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; and the other(s) of which is antibody-antigen complex to a second, third, fourth,etc. different protein. A second protein can be used, *e.g.*, as an internal control to minimize sample to sample variability by normalizing the amount of α-SNAP-25/SNAP-25 antibody-antigen complex detected to the amount of antibody-antigen complex detected for the second protein. As such, the second protein is usually one that is consistently expressed by the cell, such as a house-keeping protein. Non-limiting examples of a useful second protein, include, *e.g.*, a Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Syntaxin, cytokines. Methods of performing an immuno-based assay in a multiplex fashion are described in, *e.g.*, U. B. Nielsen and B. H. Geierstanger, *Multiplexed Sandwich Assays in Microarray Format*, J. Immunol. Methods. 290(1-2): 107-120 2004); R. Barry and M, Soloviev, *Quantitative Protein Profiling using Antibody Arrays*, Proteomics, 4(12): 3717-3726 (2004); M. M. Ling et al., *Multiplexing Molecular Diagnostics and Immunoassays using Emerging Microarray Technologies*, Expert Rev Mol Diagn. 7(1): 87-98 (2007); S. X. Leng et al., *ELISA and Multiplex Technologies for Cytokine Measurement in Inflammation and Aging Research*, J Gerontol A Biol Sci Med Sci. 63(8): 879-884 (2008), each of which is hereby incorporated by reference in its entirety.

[0140] Thus, in one embodiment, an immuno-based method of detecting BoNT/A activity practiced in a single-plex fashion by only detecting the presence of an antibody-antigen complex comprising an α-SNAP-25 antibody and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. In another embodiment, immuno-based method of detecting BoNT/A activity practiced in a multiplex fashion by concurrently detecting the presence of an antibody-antigen complex comprising an α-SNAP-25 antibody and a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond and at least one other antibody-antigen complex to a protein other than SNAP-25, such as, *e.g.*, GAPDH or Syntaxin.

[0141] Aspects of the present disclosure provide, in part, a method of determining BoNT/A immunoresistance. As used herein, the term "BoNT/A immunoresistance" means a mammal that does not fully respond to a BoNT/A therapy, or shows a reduced beneficial effect of a BoNT/A therapy because the immune response of that mammal, either directly or indirectly, reduces the efficacy of the therapy. A non-limiting example of reduced efficacy would be the presence in a mammal of at least one neutralizing α-BoNT/A antibody that binds to a BoNT/A toxin in a manner that reduces or prevents the specificity or activity of the toxin. As used herein, the term "BoNT/A therapy" means a treatment, remedy, cure,

healing, rehabilitation or any other means of counteracting something undesirable in a mammal requiring neuromodulation using a BoNT/A toxin or administering to a mammal one or more controlled doses of a medication, preparation or mixture of a BoNT/A toxin that has medicinal, therapeutic, curative, cosmetic, remedial or any other beneficial effect. BoNT/A therapy encompasses, without limitation, the use of any naturally occurring or modified fragment thereof, in any formulation, combined with any carrier or active ingredient and administered by any route of administration. An exemplary, well-known BoNT/A therapy is a BOTOX® therapy.

[0142] Aspects of the present disclosure provide, in part, a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies. As used herein, the term "test sample" refers to any biological matter that contains or potentially contains at least one α-BoNT/A antibody. An α-BoNT/A antibody can be a neutralizing anti-BoNT/A antibody or a non-neutralizing anti-BoNT/A antibody. As used herein, the term "neutralizing anti-BoNT/A antibodies" means any α-BoNT/A antibody that will, under physiological conditions, bind to a region of a BoNT/A toxin in such a manner as to reduce or prevent the toxin from exerting its effect in a BoNT/A therapy. As used herein, the term "non-neutralizing α-BoNT/A antibodies" means any α-BoNT/A antibody that will, under physiological conditions, bind to a region of a BoNT/A toxin, but not prevent the toxin from exerting its effect in a BoNT/A therapy. It is envisioned that any and all samples that can contain α-BoNT/A antibodies can be used in this method, including, without limitation, blood, plasma, serum and lymph fluid. In addition, any and all organisms capable of raising α-BoNT/A antibodies against a BoNT/A toxin can serve as a source for a sample including, but not limited to, birds and mammals, including mice, rats, goats, sheep, horses, donkeys, cows, primates and humans. Non-limiting examples of specific protocols for blood collection and serum preparation are described in, e.g., Marjorie Schaub Di Lorenzo & Susan King Strasinger, BLOOD COLLECTION IN HEALTHCARE (F.A. Davis Company, 2001); and Diana Garza & Kathleen Becan-McBride, PHLEBOTOMY HANDBOOK: BLOOD COLLECTION ESSENTIALS (Prentice Hall, 6th ed., 2002). These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein. A test sample can be obtained from an organism prior to exposure to a BoNT/A toxin, after a single BoNT/A treatment, after multiple BoNT/A toxin treatments, before onset of resistance to a BoNT/A therapy, or after onset of resistance to a BoNT/A therapy.

[0143] Aspects of the present disclosure provide, in part, a control sample. As used herein, the term "control sample" means any sample in which the presence or absence of the test sample is known and includes both negative and positive control samples. With respect to neutralizing α-BoNT/A antibodies, a negative control sample can be obtained from an individual who had never been exposed to BoNT/A and may include, without limitation, a sample from the same individual supplying the test sample, but taken before undergoing a BoNT/A therapy; a sample taken from a different individual never been exposed to BoNT/A; a pooled sample taken from a plurality of different individuals never been exposed to BoNT/A. With respect to neutralizing α-BoNT/A antibodies, a positive control sample can be obtained from an individual manifesting BoNT/A immunoresistance and includes, without limitation, individual testing

positive in a patient-based testing assays; individual testing positive in an *in vivo* bioassay; and individual showing hyperimmunity, *e.g.*, a BoNT/A vaccinated individual.

[0144] It is further foreseen that α-BoNT/A antibodies can be purified from a sample. Anti-BoNT/A antibodies can be purified from a sample, using a variety of procedures including, without limitation, Protein A/G chromatography and affinity chromatography. Non-limiting examples of specific protocols for purifying antibodies from a sample are described in, *e.g.*, ANTIBODIES: A LABORATORY MANUAL (Edward Harlow & David Lane, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1998); USING ANTIBODIES: A LABORATORY MANUAL: PORTABLE PROTOCOL NO. I (Edward Harlow & David Lane, Cold Spring Harbor Laboratory Press, 1998); and MOLECULAR CLONING, A LABORATORY MANUAL, *supra*, (2001), which are hereby incorporated by reference. In addition, non-limiting examples of antibody purification methods as well as well-characterized reagents, conditions and protocols are readily available from commercial vendors that include, without limitation, Pierce Biotechnology, Inc., Rockford, IL; and Zymed Laboratories, Inc., South San Francisco, CA. These protocols are routine procedures well within the scope of one skilled in the art.

[0145] Thus, in an embodiment, a sample comprises blood. In aspect of this embodiment, the sample comprises mouse blood, rat blood, goat blood, sheep blood, horse blood, donkey blood, cow blood, primate blood or human blood. In another embodiment, a sample comprises plasma. In an aspect of this embodiment, a test sample comprises mouse plasma, rat plasma, goat plasma, sheep plasma, horse plasma, donkey plasma, cow plasma, primate plasma or human plasma. In another embodiment, a sample comprises serum. In an aspect of this embodiment, the sample comprises mouse serum, rat serum, goat serum, sheep serum, horse serum, donkey serum, cow serum, primate serum and human serum. In another embodiment, a sample comprises lymph fluid. In aspect of this embodiment, a sample comprises mouse lymph fluid, rat lymph fluid, goat lymph fluid, sheep lymph fluid, horse lymph fluid, donkey lymph fluid, cow lymph fluid, primate lymph fluid or human lymph fluid. In yet another embodiment, a sample is a test sample. In yet another embodiment, a sample is a control sample. In aspects of this embodiment, a control sample is a negative control sample or a positive control sample.

[0146] Aspects of the present disclosure provide, in part, comparing the amount of SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond detected in step (d) to the amount of SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond detected in step (e). In an embodiment, the amount of SNAP-25 cleavage product in the test sample is higher as compared to the amount of SNAP-25 cleavage product in the control sample. In an aspect of this embodiment, a higher amount of SNAP-25 cleavage product in the test sample as compared to a positive control sample indicates a reduction in or lack of BoNT/A immunoresistance in the mammal. In another aspect of this embodiment, an equivalent amount of SNAP-25 cleavage product in the test sample as compared to a negative control sample indicates a reduction in or lack of BoNT/A immunoresistance in the mammal. In another embodiment, the amount of SNAP-25 cleavage product in

the test sample is lower as compared to the amount of SNAP-25 cleavage product in the control sample. In an aspect of this embodiment, a lower or equivalent amount of SNAP-25 cleavage product in the test sample as compared to a positive control sample indicates an increase in or presence of BoNT/A immunoresistance in the mammal. In another aspect of this embodiment, a lower amount of SNAP-25 cleavage product in the test sample as compared to a negative control sample indicates an increase in or presence of BoNT/A immunoresistance in the mammal.

[0147] It is envisioned that any and all assay conditions suitable for detecting the present of a neutralizing α-BoNT/A antibody in a sample are useful in the methods disclosed in the present specification, such as, *e.g.*, linear assay conditions and non-linear assay conditions. In an embodiment, the assay conditions are linear. In an aspect of this embodiment, the assay amount of a BoNT/A is in excess. In another aspect of this embodiment, the assay amount of a BoNT/A is rate-limiting. In another aspect of this embodiment, the assay amount of a test sample is rate-limiting.

[0148] Aspects of the present disclosure can also be described as follows:

1. A composition comprising a carrier linked to a flexible linker linked to SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

2. The composition of 1, wherein the the P_1 residue of the BoNT/A cleavage site scissile bond is glutamine or lysine.

3. The composition of 1, wherein the SNAP-25 antigen comprises SEQ ID NO: 147.

4. The composition of 1, wherein the flexible linker and the SNAP-25 antigen amino acid sequence is SEQ ID NO: 38 or SEQ ID NO: 46.

5. An isolated α-SNAP-25 antibody, wherein the isolated α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product.

6. The isolated α-SNAP-25 antibody of 5, wherein the α-SNAP-25 antibody has an association rate constant for an epitope not comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product of less than 1×10^1 M^{-1} s^{-1}; and wherein the α-SNAP-25 antibody has an equilibrium disassociation constant for the epitope of less than 0.450 nM.

7. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody has a heavy chain variable region comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 80, and SEQ ID NO: 82; and a light chain

variable region comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, and SEQ ID NO: 92.

8. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_H CDR1 of SEQ ID NO: 93, the V_H CDR1 of SEQ ID NO: 94, the V_H CDR1 of SEQ ID NO: 95, the V_H CDR1 of SEQ ID NO: 118, the V_H CDR1 of SEQ ID NO: 119, or the V_H CDR1 of SEQ ID NO: 120.

9. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_H CDR2 of SEQ ID NO: 96, the V_H CDR2 of SEQ ID NO: 97, the V_H CDR2 of SEQ ID NO: 98, the V_H CDR2 of SEQ ID NO: 99, the V_H CDR2 of SEQ ID NO: 121, the V_H CDR2 of SEQ ID NO: 122, or the V_H CDR2 of SEQ ID NO: 123.

10. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_H CDR3 of SEQ ID NO: 100, the V_H CDR3 of SEQ ID NO: 101, the V_H CDR3 of SEQ ID NO: 102, or the V_H CDR3 of SEQ ID NO: 124.

11. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_L CDR1 of SEQ ID NO: 103, the V_L CDR1 of SEQ ID NO: 104, the V_L CDR1 of SEQ ID NO: 105, the V_L CDR1 of SEQ ID NO: 106, the V_L CDR1 of SEQ ID NO: 107, the V_L CDR1 of SEQ ID NO: 125, the V_L CDR1 of SEQ ID NO: 126, the V_L CDR1 of SEQ ID NO: 127, the V_L CDR1 of SEQ ID NO: 128, or the V_L CDR1 of SEQ ID NO: 129.

12. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_L CDR2 of SEQ ID NO: 108, the V_L CDR2 of SEQ ID NO: 109, the V_L CDR2 of SEQ ID NO: 110, the V_L CDR2 of SEQ ID NO: 111, or the V_L CDR2 of SEQ ID NO: 112.

13. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises at least the V_L CDR3 of SEQ ID NO: 113, the V_L CDR3 of SEQ ID NO: 114, the V_L CDR3 of SEQ ID NO: 115, the V_L CDR3 of SEQ ID NO: 116, or the V_L CDR3 of SEQ ID NO: 117.

14. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody comprises a heavy chain variable region comprising SEQ ID NO: 93, SEQ ID NO: 121 and SEQ ID NO: 100; and a light chain variable region comprising SEQ ID NO: 105, SEQ ID NO: 110 and SEQ ID NO: 115.

15. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody selectively binds the SNAP-25 epitope of SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 147, or SEQ ID NO: 148.

16. The isolated α-SNAP-25 antibody of 5, wherein the isolated α-SNAP-25 antibody selectively binds the SNAP-25 epitope of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

17. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line is susceptible to BoNT/A intoxication by a BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

18. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line is susceptible to BoNT/A intoxication by a BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

19. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line is susceptible to BoNT/A intoxication by a BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) fixing the SNAP-25 component to a solid phase support; d) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

20. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line

can uptake BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

21. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line can uptake BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and d) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

22. A method of detecting BoNT/A activity, the method comprising the steps of: a) treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line can uptake BoNT/A; b) isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; c) fixing the SNAP-25 component to a solid phase support; d) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; and e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

23. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line is susceptible to BoNT/A intoxication; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; e) detecting the presence of an antibody-antigen complex comprising the

α-SNAP-25 antibody and the SNAP-25 cleavage product; f) repeating steps b-e with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and g) comparing the amount of antibody-antigen complex detected in step e to the amount of antibody-antigen complex detected in step f, wherein detection of a lower amount of antibody-antigen complex detected in step e relative to the amount of antibody-antigen complex detected in step f is indicative of the presence of α-BoNT/A neutralizing antibodies.

24. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line is susceptible to BoNT/A intoxication; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; f) repeating steps b-e with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and g) comparing the amount of antibody-antigen complex detected in step e to the amount of antibody-antigen complex detected in step f, wherein detection of a lower amount of antibody-antigen complex detected in step e relative to the amount of antibody-antigen complex detected in step f is indicative of the presence of α-BoNT/A neutralizing antibodies.

25. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line is susceptible to BoNT/A intoxication; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) fixing the SNAP-25 component to a solid phase support; e) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; f) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; g) repeating steps b-f with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and h) comparing the amount of antibody-antigen complex detected in step f to the amount of antibody-antigen complex detected in step g, wherein detection of a lower amount of

antibody-antigen complex detected in step f relative to the amount of antibody-antigen complex detected in step g is indicative of the presence of α-BoNT/A neutralizing antibodies.

26. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line can uptake BoNT/A; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; f) repeating steps b-e with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and g) comparing the amount of antibody-antigen complex detected in step e to the amount of antibody-antigen complex detected in step f, wherein detection of a lower amount of antibody-antigen complex detected in step e relative to the amount of antibody-antigen complex detected in step f is indicative of the presence of α-BoNT/A neutralizing antibodies.

27. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample, wherein the cell from an established cell line can uptake BoNT/A; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; e) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; f) repeating steps b-e with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and g) comparing the amount of antibody-antigen complex detected in step e to the amount of antibody-antigen complex detected in step f, wherein detection of a lower amount of antibody-antigen complex detected in step e relative to the amount of antibody-antigen complex detected in step f is indicative of the presence of α-BoNT/A neutralizing antibodies.

28. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of: a) adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies; b) treating a cell from an established cell line with the test sample,

wherein the cell from an established cell line can uptake BoNT/A; c) isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond; d) fixing the SNAP-25 component to a solid phase support; e) contacting the SNAP-25 component with an α-SNAP-25 antibody, wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product; f) detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product; g) repeating steps b-f with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and h) comparing the amount of antibody-antigen complex detected in step f to the amount of antibody-antigen complex detected in step g, wherein detection of a lower amount of antibody-antigen complex detected in step f relative to the amount of antibody-antigen complex detected in step g is indicative of the presence of α-BoNT/A neutralizing antibodies.

29. The method of 17-22 and 23-25, wherein the cell is susceptible to BoNT/A intoxication by about 500 pM or less, by about 400 pM or less, by about 300 pM or less, by about 200 pM or less, by about 100 pM or less of a BoNT/A.

30. The method of 20-22 and 26-28, wherein the cell can uptake about 500 pM or less, by about 400 pM or less, by about 300 pM or less, by about 200 pM or less, by about 100 pM or less of BoNT/A.

31. The method of 17-22, wherein the sample comprises about 100 ng or less, about 10 ng or less, about 1 ng or less, 100 fg or less, 10fg or less, or 1 fg or less of a BoNT/A

32. The method of 17-22, wherein the sample comprises about 100 nM or less, about 10 nM or less, about 1 nM or less, about 100 pM or less, about 10 pM or less, about 1 pM or less, about 100 fM or less, about 10 fM or less, or about 1 fM or less of a BoNT/A.

33. The method of 17-28, wherein the α-SNAP-25 antibody is the isolated α-SNAP-25 antibody of 5-16.

34. The method of 17-28, wherein the presence of an antibody-antigen complex is detected by an immuno-blot analysis, an immunoprecipitation analysis, an ELISA, or a sandwich ELISA

35. The method of 17-28, wherein the immuno-based method has a signal-to-noise ratio for the lower asymptote of at least 3:1, at least 5:1, at least 10:1, at least 20:1, at least 50:1, or at least 100:1.

36. The method of 17-28, wherein the immuno-based method has a signal-to-noise ratio for the higher asymptote of at least 10:1, at least 20:1, at least 50:1, at least 100:1, at least 200:1, at least 300:1, at least 400:1, at least 500:1, or at least 600:1.

37. The method of 17-28, wherein the immuno-based method can detect the EC_{50} activity of, *e.g.*, at least 100 ng, at least 50 ng, at least 10 ng, at least 5 ng, at least 100 pg, at least 50 pg, at least 10 pg, at least 5 pg, at least 100 fg, at least 50 fg, at least 10 fg, or at least 5 fg.

38. The method of 17-28, wherein the immuno-based method can detect the EC_{50} activity of, *e.g.*, at least 10 nM, at least 5 nM, at least 100 pM, at least 50 pM, at least 10 pM, at least 5 pM, at least 100 fM, at least 50 fM, at least 10 fM, at least 5 fM, or at least 1 fM.

39. The method of 17-28, wherein the immuno-based method has an LOD of, *e.g.*, 10 pg or less, 9 pg or less, 8 pg or less, 7 pg or less, 6 pg or less, 5 pg or less, 4 pg or less, 3 pg or less, 2 pg or less, 1 pg or less of a BoNT/A

40. The method of 17-28, wherein the immuno-based method has an LOD of, *e.g.*, 100 fM or less, 90 fM or less, 80 fM or less, 70 fM or less, 60 fM or less, 50 fM or less, 40 fM or less, 30 fM or less, 20 fM or less, or 10 fM or less of a BoNT/A.

41. The method of 17-28, wherein the immuno-based method has an LOQ of, *e.g.*, 10 pg or less, 9 pg or less, 8 pg or less, 7 pg or less, 6 pg or less, 5 pg or less, 4 pg or less, 3 pg or less, 2 pg or less, 1 pg or less of a BoNT/A

42. The method of 17-28, wherein the immuno-based method has an LOQ of, *e.g.*, 100 fM or less, 90 fM or less, 80 fM or less, 70 fM or less, 60 fM or less, 50 fM or less, 40 fM or less, 30 fM or less, 20 fM or less, or 10 fM or less of a BoNT/A.

43. The method of 17-28, wherein the immuno-based method can distinguish a fully-active BoNT/A from a partially-active BoNT/A having 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, or 10% or less the activity of a fully-active BoNT/A.

EXAMPLES

Example I
Screening of Candidate Cell Lines

[0149] The following example illustrates how to identify established cell lines susceptible to BoNT/A intoxication or have BoNT/A uptake capacity required for a method of detecting BoNT/A activity disclosed in the rpesent specification.

1. Growth of Stock Culture of candidate cell lines.

[0150] To grow the cell lines, a suitable density of cells from the cell line being tested were plated in a 162 cm^2 tissue culture flask containing 30 mL of a suitable growth medium (see Table 1), and grown in a 37 °C incubator under 5% or 10% carbon dioxide until cells reached the desired density.

Table 1. Media Used in Cell Line Screening.

Cell Line	Serum Growth Media Composition
Kelly SiMa	RPMI 1640, 10% fetal bovine serum, 1% Penicillin-Streptomycin, 2 mM L-Glutamine
NB69	RPMI 1640, 15% fetal bovine serum, 1% Penicillin-Streptomycin
CHP-126	RPMI 1640, 20% fetal bovine serum, 1% Penicillin-Streptomycin
N4TG3	RPMI 1640, 10% fetal bovine serum, 1% Penicillin-Streptomycin, 100 µM 6-thioguanine
MHH-NB-11	RPMI 1640, 10% fetal bovine serum, 1% Penicillin-Streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids
PC12	RPMI 1640, 5% heat-inactivated fetal bovine serum, 10% equine serum, 2 mM GlutaMAX™, 10 mM HEPES, 1 mM sodium pyruvate, 1% Penicillin-Streptomycin
N18TG2	DMEM (11885-084, Gibco), 10% fetal bovine serum, 1% Penicillin-Streptomycin, 100 µM 6-thioguanine
N1E-115 N18 ND8/34 NG108-15 NG115-401L NS20Y SK-N-SH	90 % DMEM, 10% heat-inactivated fetal bovine serum, 2 mM Glutamine, 2 mM glucose
SK-N-DZ SK-N-F1	90% DMEM, 10% heat-inactivated fetal bovine serum, 4 mM Glutamine, 4 mM glucose, 0.1 mM non-essential amino acids, 1.5 g/L $NaHCO_3$
BE(2)-C BE(2)-M17 CHP-212 LA-1-55n LA-N-1 MC-1XC SK-N-BE(2) SH-SY5Y	EMEM(11090-081, Gibco), Ham's F12 (11765-054, Gibco), 10% heat-inactivated fetal bovine serum, 2 mM Glutamine, 0.1 mM non-essential amino acids,
NB4 1A3	Ham's F10 (12471-017, Gibco), 2.5% heat-inactivated fetal bovine serum, 15% heat-inactivated horse serum, 2 mM Glutamine
Neuro-2a	EMEM, 10% heat-inactivated fetal bovine serum, 2 mM Glutamine, 0.1 mM non-essential amino acids, 1.5 g/L $NaHCO_3$, 1 mM Sodium pyruvate

2. Single-dose screening of candidate cell lines using 1 nM BoNT/A.

[0151] One parameter tested to improve the sensitivity of a cell-based assay was to identify suitable cell lines that exhibited a good capacity to uptake a Clostridial neurotoxin and adhere to a substrate surface. Initially, cell lines were tested for their ability to uptake 1 nM BoNT/A and their ability to attach to a surface. To determine whether a cell line was able to uptake 1 nM BoNT/A, a suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of an appropriate serum growth medium (Table 1). The cells were grown in a 37 °C

incubator under 5% carbon dioxide until cells reached the desired density (approximately 18 to 24 hours). The growth media was aspirated from each well and replaced with either 1) fresh growth media containing no toxin (untreated cell line) or 2) fresh growth media containing 1 nM of a BoNT/A complex (treated cell line). After an overnight incubation, the cells were washed by aspirating the growth media and rinsing each well with 200 µl of 1 x PBS. To harvest the cells, the 1 x PBS was aspirated, the cells were lysed by adding 50 µl of 2 x SDS Loading Buffer, the lysate was transferred to a clean test tube and the sample was heated to 95 °C for 5 minutes.

[0152] To detect for the presence of both uncleaved SNAP-25 substrate and cleaved SNAP-25 products, an aliquot from each harvested sample was analyzed by Western blot. In this analysis, a 12 µl aliquot of the harvested sample was separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 12% Bis-Tris precast polyacrylamide gels (Invitrogen Inc., Carlsbad, CA) under denaturing, reducing conditions. Separated peptides were transferred from the gel onto polyvinylidene fluoride (PVDF) membranes (Invitrogen Inc., Carlsbad, CA) by Western blotting using a Trans-Blot® SD semi-dry electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Hercules, CA). PVDF membranes were blocked by incubating at room temperature for 2 hours in a solution containing Tris-Buffered Saline (TBS) (25 mM 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloric acid (Tris-HCl)(pH 7.4), 137 mM sodium chloride, 2.7 mM potassium chloride), 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate), 2% Bovine Serum Albumin (BSA), 5% nonfat dry milk. Blocked membranes were incubated at 4 °C for overnight in TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate), 2% BSA, and 5% nonfat dry milk containing either 1) a 1:5,000 dilution of an α-SNAP-25 mouse monoclonal antibody as the primary antibody (SMI-81; Sternberger Monoclonals Inc., Lutherville, MD); or 2) a 1:5,000 dilution of S9684 α-SNAP-25 rabbit polyclonal antiserum as the primary antibody (Sigma, St. Louis, MO). Both α-SNAP-25 mouse monoclonal and rabbit polyclonal antibodies can detect both the uncleaved SNAP-25 substrate and the SNAP-25 cleavage product, allowing for the assessment of overall SNAP-25 expression in each cell line and the percent of SNAP-25 cleaved after BoNT/A treatment as a parameter to assess the amount of BoNT/A uptake. Primary antibody probed blots were washed three times for 15 minutes each time in TBS, TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Washed membranes were incubated at room temperature for 2 hours in TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate), 2% BSA, and 5% nonfat dry milk containing either 1) a 1:10,000 dilution of goat polyclonal anti-mouse immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) as a secondary antibody; or 2) a 1:10,000 dilution of goat polyclonal anti-rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) as a secondary antibody. Secondary antibody-probed blots were washed three times for 15 minutes each time in TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Signal detection of the labeled SNAP-25 products were visualized using the ECL Plus™ Western Blot Detection System (GE Healthcare, Amersham Biosciences, Piscataway, NJ) and the membrane was imaged and the percent of cleaved quantified with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (GE Healthcare,

Amersham Biosciences, Piscataway, NJ). The choice of pixel size (100 to 200 pixels) and PMT voltage settings (350 to 600, normally 400) depended on the individual blot. Table 2 indicates the cell lines where a SNAP-25 cleavage product was detected when treated with 1 nM BoNT/A. The following cell lines exhibited both an uptake of 1 nM BoNT/A and appropriate attachment to a substrate surface: BE(2)-M17, IMR-32, Kelly, LA1-55n, N1E-115, N4TG3, N18, Neuro-2a, NG108-15, PC12, SH-SY5Y, SiMa and SK-N-BE(2)-C.

[0153] To determine whether a cell line was able to attach to a surface, a suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of an appropriate growth media (Table 1). The cells were grown in a 37 °C incubator under 5% carbon dioxide until cells reach the desired density (approximately 18 to 24 hours). Cell attachment was assessed by the percentage of cells that adhered to the bottom well surface of the tissue plate relative to the total number of cells seeded. Cell lines CHP-126, IMR-32, LA-N-1, MC-IXC, NG115-401L, SK-N-BE(2)-C, SK-N-F1 and SK-N-MC were deemed unsuitable because each cell line exhibited less than 50% attachment (Table 2). All other cells lines tested exhibited suitable cell attachment characteristics (Table 2).

Table 2. Single-Dose Screening of Candidate Cell Lines Using 1 nM BoNT/A.

Cell Line	Description	Source	1 nM BoNT/A Uptake	Attachment
BE(2)-C	Human neuroblastoma	ATCC CRL-2268	No	>60%
BE(2)-M17	Human neuroblastoma	ATCC CRL-2267	Yes	>60%
CHP-126	Human neuroblastoma	DSMZ ACC 304	No	<50%
CHP-212	Human neuroblastoma	ATCC CRL-2273	No	>60%
HCN-1a	Brain cortical neuron	ATCC CRL-10442	No	>60%
HCN-2	Brain cortical neuron	ATCC CRL-10742	No	>60%
IMR-32	Human neuroblastoma	ATCC CRL-127	Yes	<50%
Kelly	Human neuroblastoma	ECACC 92110411	Yes	>60%
Kelly	Human neuroblastoma	DSMZ ACC 355	Yes	>60%
LA1-55n	Human neuroblastoma	ECACC 06041203	Yes	>60%
LA-N-1	Human neuroblastoma	ECACC 06041201	—	<25%
MC-IXC	Human neuroepithelioma	ATCC CRL-2270	—	<25%
MHH-NB-11	Human neuroblastoma	DSMZ ACC 157	No	>60%
N1E-115	Mouse neuroblastoma	ATCC CCL-2263	Yes	>60%
N4TG3	Mouse neuroblastoma	DSMZ ACC 101	No	>60%
N18TG2	Mouse neuroblastoma	DSMZ ACC 103	No	>60%
NB4 1A3	Mouse neuroblastoma	ECACC 89121405	No	>60%
ND3	Mouse neuroblastoma/primary neonatal rat DRG hybrid	ECACC 92090901	No	>60%

ND7/23	Mouse neuroblastoma/primary rat DRG hybrid	ECACC 92090903	No	>60%
ND8	Mouse neuroblastoma/primary neonatal rat DRG hybrid	**ATCC**	No	>60%
ND8/34	Mouse neuroblastoma	ECACC 92090904	No	>60%
ND15	Mouse neuroblastoma/primary neonatal rat DRG hybrid	ECACC 92090907	No	>60%
ND27	Mouse neuroblastoma/primary rat DRG hybrid	ECACC 92090912	No	>60%
NB69	Human neuroblastoma	ECACC 99072802	No	>60%
NDC	Mouse neuroblastoma/primary neonatal rat DRG hybrid	ECACC 92090913	No	>60%
Neuro-2a	Mouse neuroblastoma	ATCC CCL-131	Yes	>60%
NG108-15	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112302	Yes	>60%
NG115-401L	Mouse neuroblastoma/rat glioma hybrid	ECACC 87032003	No	<50%
NS20Y	Mouse neuroblastoma	DSMZ ACC 94	No	>60%
PC12	Rat pheochromocytoma	ATCC CRL-1721	Yes	>60%
SH-SY5Y	Human neuroblastoma	ATCC CRL-2266	Yes	>60%
SiMa	Human neuroblastoma	DSMZ ACC 164	Yes	>60%
SK-N-BE(2)-C	Human neuroblastoma	ATCC CRL-2271	Yes	<50%
SK-N-AS	Human neuroblastoma	ATCC CRL-2137	No	>60%
SK-N-DZ	Human neuroblastoma	ATCC CRL-2149	No	>60%
SK-N-F1	Human neuroblastoma	ATCC CRL-2142	No	<50%
SK-N-MC	Human neuroblastoma	ATCC HTB-10	—	<25%
SK-N-SH	Human neuroblastoma	ECACC 86012802	No	>60%
TE 189.T	Spinal cord	ATCC CRL-7947	No	>60%

Example II

Evaluation of Growth Conditions on Neurotoxin Uptake in Candidate Cell Lines

[0154] The following example illustrates how to determine growth conditions for established cell lines that maximize susceptible to BoNT/A intoxication or have BoNT/A uptake capacity.

1. Effects of cell differentiation on neurotoxin uptake of candidate cell lines.

[0155] To determine whether cell differentiation improved neurotoxin uptake, cell lines exhibiting uptake of 1 nM BoNT/A were transferred into serum-free medium to induced differentiation. A suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of a serum-free medium containing Minimum Essential Medium with 2 mM GlutaMAX™ I with Earle's salts, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES, 1 mM Sodium

Pyruvate, 100 units/mL Penicillin, and 100 µg/mL Streptomycin. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 2 to 3 days). As a control, a suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of an appropriate growth medium (Table 1). These undifferentiated control cells were grown in a 37 °C incubator under 5% carbon dioxide until cells reach the desired density (approximately 18 to 24 hours). The media from both differentiated and undifferentiated control cultures was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.1 nM, 0.3 nM, or 1 nM of a BoNT/A complex. After an overnight incubation, the cells were washed and harvested as described in Example I.

[0156] To detect for the presence of cleaved SNAP-25 products, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that harvested samples are separated by SDS-PAGE using 12 % 26-well Criterion gels (Bio-Rad Laboratories, Hercules, CA), and the rabbit polyclonal α-SNAP-25_{197} antibody serum was used as the primary antibody (see Example IV). Table 3 indicates the cell lines that exhibited a SNAP-25 cleavage product when treated with 0.1 nM BoNT/A. Of the cell lines tested, only the SiMa and Neuro-2a cell lines exhibited an uptake of 0.1 nM BoNT/A in the undifferentiated state. However, besides SiMa and Neuro-2a, the cell lines N18, LA1-55n, PC12, and SH-SY5Y all exhibited an uptake of 0.1 nM BoNT/A in the differentiated state.

Table 3. Effects of Cell Differentiation on Neurotoxin Uptake of Candidate Cell Lines.

Cell Line	Description	Source	0.1 nM BoNT/A Uptake	
			Undifferentiated	Differentiated
BE(2)-M17	Human neuroblastoma	ATCC CRL-2267	No	No
Kelly	Human neuroblastoma	DSMZ ACC 355	No	No
LA1-55n	Human neuroblastoma	ECACC 06041203	No	Yes
N1E-115	Mouse neuroblastoma	ATCC CCL-2263	No	Not Tested
N4TG3	Mouse neuroblastoma	DSMZ ACC 101	No	Not Tested
N18	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112301	No	Yes
Neuro-2a	Mouse neuroblastoma	ATCC CCL-131	Yes	Yes
NG108-15	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112302	No	Not Tested
PC12	Rat pheochromocytoma	ATCC CRL-1721	No	Yes
SH-SY5Y	Human neuroblastoma	ATCC CRL-2266	No	Yes
SiMa	Human neuroblastoma	DSMZ ACC 164	Yes	Yes
SK-N-BE(2)-C	Human neuroblastoma	ATCC CRL-2271	No	Not Tested

2. Effects of ganglioside treatment on neurotoxin uptake of differentiated candidate cell lines.

[0157] To determine whether treatments improving low-affinity binding of neurotoxin could improve neurotoxin uptake, differentiated cell lines exhibiting uptake of 1 nM BoNT/A were treated with ganglioside GT1b. A suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing serum-free medium as described above, with or without 25 µg/mL GT1b (Alexis Biochemicals, San Diego, CA). These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria as described above. The media was aspirated from each well and replaced with fresh serum-free media containing either 0 (untreated sample), 1.9 pM, 3.7 pM, 7.4 pM, 14.8 pM, 29.7 pM, 59.4 pM, 118.8 pM, 237.5 pM, 574 pM, 950 pM, and 1900 pM of a BoNT/A complex. The cell lines were incubated at two different times, 24 hours and 48 hours. After toxin incubation, the cells were washed and harvested as described in Example I.

[0158] To detect for the presence of cleaved SNAP-25 products, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that harvested samples are separated by SDS-PAGE using 12% 26-well Criterion gels (Bio-Rad Laboratories, Hercules, CA), and the rabbit polyclonal α-SNAP-25_{197} antibody serum was used as the primary antibody (see Example IV). Table 4 indicates the effects of gangliosides treatment on the ability of differentiated cell lines to uptake BoNT/A. These results indicate the lowest concentration of BoNT/A that will produce a detectable band of SNAP-25 cleavage product in the Western blot.

Table 4. Effects of GangliosideTreatment on Neurotoxin Uptake of Candidate Cell Lines.

Cell Line	Description	Source	BoNT/A Uptake	
			24 Hour Incubation	48 Hour Incubation
BE(2)-M17	Human neuroblastoma	ATCC CRL-2267	237.5 pM	118.8 pM
Kelly	Human neuroblastoma	DSMZ ACC 355	Not Tested	Not Tested
LA1-55n	Human neuroblastoma	ECACC 06041203	15 pM	7.4 pM
N1E-115	Mouse neuroblastoma	ATCC CCL-2263	Not Tested	Not Tested
N4TG3	Mouse neuroblastoma	DSMZ ACC 101	Not Tested	Not Tested
N18	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112301	14.8 pM	7.4 pM
Neuro-2a	Mouse neuroblastoma	ATCC CCL-131	7.4 pM	7.4 pM
NG108-15	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112302	Not Tested	Not Tested
PC12	Rat pheochromocytoma	ATCC CRL-1721	7.4 pM	7.4 pM
SH-SY5Y	Human neuroblastoma	ATCC CRL-2266	Not Tested	Not Tested
SiMa	Human neuroblastoma	DSMZ ACC 164	1.9 pM	1.9 pM
SK-N-BE(2)-C	Human neuroblastoma	ATCC CRL-2271	Not Tested	Not Tested

3. Development of serum-free media with cell differentiating properties that enhanced neurotoxin uptake of candidate cell lines.

[0159] To determine whether treatment improvements that induce cell differentiation could improve neurotoxin uptake, SiMa, Neuro-2a and PC12 cell lines were grown in various serum-free medium to induced differentiation. A suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of various test serum-free medium. Parameters tested were 1) the effect of different basal media on BoNT/A uptake (MEM and RPMI 1649); 2) the effect of the presence or absence of neurotrophic factors on BoNT/A uptake (N2 supplement and B27 supplement); 3) the effect of the presence or absence of differentiation factors on BoNT/A uptake (retinoic acid and nerve growth factor); and 4) the effect of the presence or absence of serum on BoNT/A uptake (serum-free media and reduced serum media). As a control, a suitable density of cells from a stock culture of the cell line being tested was plated into the wells of 24-well tissue culture plates containing 1 mL of a control serum-free media (Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES, 1 mM Sodium Pyruvate, 100 units/mL Penicillin, and 100 µg/mL Streptomycin). These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 2 to 3 days). The media was aspirated from each well and replaced with fresh serum-free media containing either 0 (untreated sample), 0.005 pM, 0.015 pM, 0.05 pM,, 0.14 pM, 0.42 pM, 1.2 pM, 3.7 pM, 11 pM, 33 pM, 100 pM and 300 pM of a BoNT/A complex. In addition, the differentiated cells were treated with BoNT/A for 24 hrs followed by a media change and 48 hrs incubation in fresh media without toxin to allow for the accumulation of SNAP-25 cleavage product. The cells were then washed and harvested as described in Example I.

Table 5. Serum Free Media Used for Differentiating Cell Lines.

Cell Line	Test Serum Free Media Composition
LA1-55n	Minimum Essential Medium with 2 mM GlutaMAX™ I with Earle's salts, 0.1 mM Non-Essential Amino-Acids, 10 mM HEPES, 1x N2 supplement, and 1 x B27 supplement
Neuro-2a	Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES
PC12	RPMI 1640, 2 mM GlutaMAX™, 1 x B27 supplement, 1 x N2 supplement, 10 mM HEPES, 1 mM sodium pyruvate, 1% Penicillin-Streptomycin and 50 ng/mL Nerve Growth Factor
SiMa	Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES

[0160] To detect for the presence of a SNAP-25 cleavage product, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that harvested samples are separated by SDS-PAGE using 12 % 26-well Criterion gels (Bio-Rad Laboratories, Hercules, CA), and an α-SNAP-25 rabbit polyclonal antibody serum was used (see Example IV). The most optimized media determined for each cell line is shown in Table 5. Table 6 indicates the lowest amount of a SNAP-25 cleavage product detected when the cell lines were grown in this optimized serum-free medium. Use of the optimized serum-free medium resulted in the detection of BoNT/A activity signals with acceptable signal-to-noise ratios in LA1-55n, Neuro-2a, PC-12, and SiMa cell lines (FIG. 2). For example, optimized differentiation conditions resulted in a 5-fold increase in SNAP-25 cleavage product detection as

compared to the control serum-free media for Neuro-2a and PC12 cells, and almost 50-fold for SiMa cells. In addition, a minimal signal to noise ratio of 3:1 for the lower asymptote and 10:1 for the upper asymptote is required to develop a robust assay amenable for validation. With the exception of LA-1-55n, all optimized cell lines provided a signal to noise ratio for the lower asymptote of at least 3:1 when the signal detected from the 1.2 pM dose was compared to the background signal of 0 pM BoNT/A (FIG. 2). In addition, all optimized cell lines provided a signal to noise ratio for the upper asymptote of at least 100:1 when the signal from the 300 pM dose was compared to the background signal of 0 pM BoNT/A (FIG. 2). These results indicate that any of these cell lines could be used to develop an immuno-based method for detecting BoNT/A activity as disclosed in the present specification because the assay was detecting the presence of pM amounts of BoNT/A.

Table 6. Effects of Optimized Serum-Free Media on Neurotoxin Uptake of Candidate Cell Lines.

Cell Line	Description	Source	BoNT/A Uptake	
			Control Serum-Free Media	Optimized Serum-Free Media
BE(2)-M17	Human neuroblastoma	ATCC CRL-2267	Not Tested	Not Tested
Kelly	Human neuroblastoma	DSMZ ACC 355	Not Tested	Not Tested
LA1-55n	Human neuroblastoma	ECACC 06041203	7.4 pM	3.7 pM
N1E-115	Mouse neuroblastoma	ATCC CCL-2263	Not Tested	Not Tested
N4TG3	Mouse neuroblastoma	DSMZ ACC 101	Not Tested	Not Tested
N18	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112301	Not Tested	Not Tested
Neuro-2a	Mouse neuroblastoma	ATCC CCL-131	3.7 pM	0.8 pM
NG108-15	Mouse neuroblastoma/rat glioma hybrid	ECACC 88112302	Not Tested	Not Tested
PC12	Rat pheochromocytoma	ATCC CRL-1721	2.0 pM	0.42 pM
SH-SY5Y	Human neuroblastoma	ATCC CRL-2266	Not Tested	Not Tested
SiMa	Human neuroblastoma	DSMZ ACC 164	0.23 pM	0.005 pM
SK-N-BE(2)-C	Human neuroblastoma	ATCC CRL-2271	Not Tested	Not Tested

Example III

Development of α-SNAP-25 Monoclonal Antibodies that Selectively Bind a SNAP-25 Epitope Having a Free Carboxyl-terminus at the P_1 Residue of the BoNT/A Cleavage Site Scissile Bond

[0161] The following example illustrates how to make α-SNAP-25 monoclonal antibodies that can selectively bind to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

1. Generation of α-SNAP-25 monoclonal antibodies.

[0162] To develop monoclonal α-SNAP-25 antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, the 13-residue peptide CDSNKTRIDEANQ$_{COOH}$ (SEQ ID NO: 38) was designed as a SNAP-25 cleavage product antigen. This peptide comprises a flexible linker region and a N-terminal Cysteine residue for conjugation to KLH and amino acids 186-197 of human SNAP-25 (SEQ ID NO: 5) with a carboxylated C-terminal glutamine (SEQ ID NO: 38). The generation of monoclonal antibodies to well-chosen, unique peptide sequences provides control over epitope specificity, allowing the identification of a particular subpopulation of protein among a pool of closely related isoforms. Blast searches revealed that this peptide has high homology only to SNAP-25 and almost no possible cross-reactivity with other proteins in neuronal cells. The sequence was also carefully scrutinized by utilizing computer algorithms to determine hydropathy index, protein surface probability, regions of flexibility, and favorable secondary structure, followed by proper orientation and presentation of the chosen peptide sequence. The peptide was synthesized and conjugated to Keyhole Limpet Hemocyanin (KLH) to increase immunogenicity. Six Balb/c mice were immunized with this peptide, and after three immunizations in about eight weeks, the mice were bled for testing. The blood was allowed to clot by incubating at 4 °C for 60 minutes. The clotted blood was centrifuged at 10,000x g at 4 °C for 10 minutes to pellet the cellular debris. The resulting serum sample was dispensed into 50 µl aliquots and stored at -20 °C until needed.

[0163] A similar strategy based on other SNAP-25 antigens disclosed in the present specification is used to develop α-SNAP-25 monoclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. For example, the SNAP-25 antigen of SEQ ID NO: 45 can be conjugated to KLH instead of the SNAP-25 antigen of SEQ ID NO: 38. As another example, the amino acids 186-197 of human SNAP-25 from the SNAP-25 antigen of SEQ ID NO: 38 can be replaced with SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44.

2. Screening for the presence of α-SNAP-25 monoclonal antibodies.

[0164] To determine the presence of an α-SNAP-25 monoclonal antibody that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, comparative ELISA and cell-based cleavage assay were performed using the extracted mouse serum. For comparative ELISA, two fusion proteins were constructed: BirA-HisTag®-SNAP-25$_{134-197}$ of SEQ ID NO: 48 and the BirA-HisTag®-SNAP-25$_{134-206}$ of SEQ ID NO: 49. BirA-HisTag®-SNAP-25$_{134-197}$ comprised a naturally-biotinylated 16 amino acid BirA peptide of SEQ ID NO: 50 amino-terminally linked to a SNAP-25 peptide comprising amino acids 134-197 of SEQ ID NO: 5. BirA-HisTag®-SNAP-25$_{134-206}$ comprised a naturally-biotinylated 16 amino acid BirA peptide of SEQ ID NO: 50 amino-terminally linked to a SNAP-25 peptide comprising amino acids 134-206 of SEQ ID NO: 5. These two substrates were suspended in 1 x PBS at a concentration of 10 µg/mL BirA-HisTag®-SNAP-25$_{134-197}$ and the BirA-HisTag®-SNAP-25$_{134-206}$.

The BirA-HisTag®-SNAP-$25_{134-197}$ and the BirA-HisTag®-SNAP-$25_{134-206}$ were coated onto separate plates by adding approximately 100 µl of the appropriate Substrate Solution and incubating the plates at room temperature for one hour. Washed plates were incubated at 37 °C for one hour in 0.5% BSA in 1 x TBS containing a 1:10 to 1:100 dilution of an antibody-containing serum derived from one of the six immunized mice (Mouse 1, Mouse 2, Mouse 3, Mouse 4, Mouse 5, and Mouse 6). Primary antibody probed plates were washed four times for 5 minutes each time in 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Washed plates were incubated at 37 °C for 1 hour in 1 x TBS containing a 1:10,000 dilution of goat polyclonal anti-mouse IgG antibody conjugated to Horseradish peroxidase (Pierce Biotechnology, Rockford, IL) as a secondary antibody. Secondary antibody-probed plates were washed four times in 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Chromogenic detection of the labeled SNAP-25 products were visualized by chromogenic detection using ImmunoPure TMB substrate kit (Pierce Biotechnology, Rockford, IL). The development of a yellow color in the BirA-HisTag®-SNAP-$25_{134-197}$ coated plates, but not the BirA-HisTag®-SNAP-$25_{134-206}$ coated plates, indicated that the α-SNAP-25 antibody preferentially recognized the SNAP-25_{197} cleavage product. The resulted indicated that of the six mice used for immunization, three mice (Mouse 2, Mouse 3, and Mouse 4) had higher titers and more specificity towards a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

[0165] These results were confirmed using an ELISA light chain activity assay. A 96-well Reacti-Bind Streptavidin coated plates (Pierce Biotechnology, Rockford, IL) were prepared by adding approximately 100 µl of the following Substrate Solution: Rows A-C were coated with 100 µL of BirA-HisTag®-SNAP-$25_{134-197}$ at twelve different concentrations; Rows D-H were coated with 100 µL of BirA-HisTag®-SNAP-$25_{134-206}$ at 10 µg/mL. The plates were washed by aspirating the Substrate Solution and rinsing each well three times with 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Dilutions of BoNT/A were pre-reduced at 37 °C for 20 minutes in BoNT/A Incubation Buffer (50 mM HEPES, pH 7.4, 1% fetal bovine serum, 10 µM $ZnCl_2$, 10 mM dithiothrietol) and 100 µl of the pre-reduced BoNT/A was added to the substrate-coated plates and incubated at 37 °C for 90 minutes. BoNT/A treated plates were washed by aspirating the BoNT/A Incubation Buffer and rinsing each plate three times with 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Washed plates were incubated at 37 °C for one hour in 0.5% BSA in 1 x TBS containing a 1:10 to 1:100 dilution of the antibody-containing serum being tested. Primary antibody probed plates were washed four times for 5 minutes each time in 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Washed plates were incubated at 37 °C for 1 hour in 1 x TBS containing a 1:10,000 dilution of goat polyclonal anti-mouse IgG antibody conjugated to Horseradish peroxidase (Pierce Biotechnology, Rockford, IL) as a secondary antibody. Secondary antibody-probed plates were washed four times in 200 µl TBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). Chromogenic detection of the labeled SNAP-25 products were visualized by chromogenic detection using ImmunoPure TMB substrate kit (Pierce Biotechnology, Rockford, IL). The development of a yellow color, which correlated with the presence of the SNAP-25_{197} cleavage product was detected in BoNT/A treated samples, but not untreated controls, using antibody-

containing serum derived from all six immunized mice (Mouse 1, Mouse 2, Mouse 3, Mouse 4, Mouse 5, and Mouse 6). Thus, the comparative ELISA analysis indicated that of the mice used for immunization, three mice (Mouse 2, Mouse 3, and Mouse 4) had higher titers and more specificity towards a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

[0166] For cell-based cleavage assay, a suitable density of PC12 cells were plated into 60 mm^2 tissue culture plates containing 3 mL of an appropriate serum medium (Table 1). The cells were grown in a 37 °C incubator under 5% carbon dioxide until cells reached the appropriate density. A 500 µL transfection solution was prepared by adding 250 µL of OPTI-MEM Reduced Serum Medium containing 15 µL of LipofectAmine 2000 (Invitrogen Inc., Carlsbad, CA) incubated at room temperature for 5 minutes to 250 µL of OPTI-MEM Reduced Serum Medium containing 10 µg of a pQBI-25/GFP-BoNT/A-LC expression construct (SEQ ID NO: 51). The pQBI-25/GFP-BoNT/A-LC expression construct comprises a pQBI-25 expression vector (Qbiogene Inc., Carlsbad, CA) whose promoter elements are functionally linked to a polynucleotide encoding the GFP-BoNT/A light chain of SEQ ID NO: 52. This transfection mixture was incubated at room temperature for approximately 20 minutes. The media was replaced with fresh unsupplemented media and the 500 µL transfection solution was added to the cells. The cells were then incubated in a 37 °C incubator under 5% carbon dioxide for approximately 6 to 18 hours. The cells were washed and harvested as described in Example II. To detect for the presence of the cleaved SNAP-25_{197} product, an aliquot from each harvested sample was analyzed by Western blot as described in Example II, except that the primary antibody used was a 1:1,000 dilution of the antibody-containing serum and the secondary antibody used was a 1:20,000 of mouse α-IgG Horseradish Peroxidase (Pierce Biotechnology, Rockford, IL). A single band corresponding to the SNAP-25_{197} cleavage product was detected in BoNT/A treated samples, but not untreated controls, using antibody-containing serum derived from three mice (Mouse 2, Mouse 3, and Mouse 4). Thus, the cell-based cleavage assay indicated that of the mice used for immunization, three mice (Mouse 2, Mouse 3, and Mouse 4) had higher titers and more specificity towards a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

3. Production of hybridomas.

[0167] To make hybridomas producing α-SNAP-25 monoclonal antibodies that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, the spleen from Mouse 2 was harvested three days subsequent to a final "booster" immunization and the spleen cells were fused with myeloma cells P3-X63 Ag8.653 using standard hybridoma protocols. These cells were plated into five 96-well plates and hybrids were selected using HAT medium. Within 8-14 days after fusion, the first screening of the approximately 480 parent clones was carried out using comparative ELISA with the BirA-HisTag®-SNAP-$25_{134-197}$ and the BirA-HisTag®-SNAP-$25_{134-206}$ peptides coated in two separate plates. The comparative ELISA provided a quick screen method to identify hybridomas producing antibodies specific for the cleaved SNAP-25_{197}. The top 18 clones were subjected to further

screening using the cell-based cleavage assay described above and immunostaining of LC/A transfected cells. (Table 7).

Table 7. Analysis of Supernatants Containing α-SNAP-25 Monoclonal Antibody						
Clone	Comparative ELISA				Cell-Based Assay	
	OD SNAP-25_{197}	OD SNAP-25_{206}	$Ratio_{197/206}$	$Ratio_{206/197}$	SNAP-25_{197}	SNAP-25_{206}
1D3	1.805	0.225	8.02	0.13	+++	–
1F12	0.365	0.093	3.92	0.25	–	–
1G10	0.590	0.137	4.31	0.23	++	–
1H1	0.335	0.121	2.77	0.36	–	–
1H8	0.310	0.302	1.03	0.97	+	–
2C9	0.139	0.274	0.51	1.97	–	–
2E2	0.892	0.036	24.78	0.04	++	–
2E4	0.228	0.069	3.30	0.30	+	–
2F11	1.095	1.781	0.61	1.63	–	–
3C1	1.268	0.053	23.92	0.04	++	–
3C3	0.809	0.052	15.56	0.06	++	–
3E1	0.086	0.155	0.55	1.80	0	–
3E8	2.048	0.053	38.64	0.03	+++	–
3G2	0.053	0.158	0.34	2.98	–	–
4D1	0.106	0.218	0.49	2.06	–	–
4G6	0.061	0.159	0.38	2.61	–	–
5A5	0.251	0.106	2.37	0.42	+	–
5F11	0.243	0.061	3.98	0.25	–	–

[0168] Clones 1D3, 1G10, 2E2, 3C1, 3C3, and 3E8 were further cloned by limiting dilution because the conditioned media produced by these clones comprised α-SNAP-25 antibodies with a preferential binding specificity having a $ratio_{197/206}$ of at least 4:1 for the SNAP-25_{197} cleavage product relative to the SNAP-25_{206} uncleaved substrate and detected the SNAP-25_{197}-cleavage product using the cell-based cleavage assay and the immunostaining of PC12 cells transfected with GFP-LC/A. Similarly clones 2C9, 2F11, 3G2, 4D1 and 4G6 were further cloned by limiting dilution because the conditioned media produced by these clones comprised α-SNAP-25 antibodies with a preferential binding specificity having a $ratio_{206/197}$ of at least 1.5:1 for the SNAP-25_{206} uncleaved substrate relative to the SNAP-25_{197} cleavage product and detected the SNAP-25_{206}-uncleaved substrate using the cell-based cleavage assay. These single-cell derived clones were screened again using comparative ELISA, cell-based cleavage, and immunostaining to confirm their affinity and specificity, and the antibodies were isotyped using standard procedures. Ascites were produced from clones 1D3B8 (IgM.k), 1G10A12 (IgG3.k), 2C9B10 (IgG3.k), 2E2A6 (IgG3.k), 2F11B6 (IgM.k), 3C1A5 (IgG2a.k), and 3C3E2 (IgG2a.k). Clone 3E8 stopped producing antibodies during the cloning process and could not be further evaluated.

4. Evaluation of binding specificity of α-SNAP-25 monoclonal antibodies.

[0169] To evaluate binding specificity of an α-SNAP-25 monoclonal antibody that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, ascites from clones 1D3B8, 1G10A12, 2C9B10, 2E2A6, 2F11B6, 3C1A5, and 3C3E2 were used to detect SNAP-25 cleavage product using the cell-based activity assay, immunocytochemistry and immunoprecipitation.

[0170] For the cell-based activity assay, binding specificity was determined by analyzing the ability of α-SNAP-25 antibody-containing ascites to detect the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product by Western blot analysis. A suitable density of PC12 cells were plated into 60 mm^2 tissue culture plates containing 3 mL of an appropriate serum medium, grown in a 37 °C incubator under 5% carbon dioxide until an appropriate cell density was reached, and transfected with the either a transfection solution lacking the pQBI-25/GFP-BoNT/A-LC expression construct (untransfected cells) or a transfection solution containing the pQBI-25/GFP-BoNT/A-LC expression construct (transfected cells) as described above. The cells were washed and harvested as described in Example I. To detect for the presence of both the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that the primary antibody used was a 1:100 dilution of the α-SNAP-25 monoclonal antibody-containing ascites and the secondary antibody used was a 1:20,000 of α-mouse IgG conjugated to Horseradish Peroxidase (Pierce Biotechnology, Rockford, IL). In addition, three commercially available mouse α-SNAP-25 monoclonal antibodies were tested. SMI-81 (Sternberger Monoclonals Inc., Lutherville, MD), an α-SNAP-25 antibody the manufacturer indicates detects both the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product, was used at a 15,000 dilution according to the manufacturer's recommendations. MC-6050 (Research & Diagnostic Antibodies, Las Vegas, NV), an α-SNAP-25 antibody the manufacturer indicates detects both the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product, was used at a 1:100 dilution according to the manufacturer's recommendations. MC-6053 (Research & Diagnostic Antibodies, Las Vegas, NV), an α-SNAP-25 antibody the manufacturer indicates detects only the cleaved SNAP-25_{197} product, was used at a 1:100 dilution according to the manufacturer's recommendations.

[0171] Table 8 indicates the α-SNAP-25 antibody-containing ascites that detected only the SNAP-25_{197} cleavage product. The cell-based cleavage assay indicated that ascites produced from clones 1D3B8, 2C9B10, 2E2A6, 3C1A5, and 3C3E2 synthesize an α-SNAP-25 monoclonal antibody having high binding specificity for the SNAP-25_{197} cleavage product that allows for the selective recognition of this cleavage product relative to the SNAP-25_{206} uncleaved substrate. Commercial antibody SMI-81 detected the SNAP-25_{206} uncleaved substrate, but only poorly recognized the SNAP-25_{197} cleavage product (Table 8). Surprisingly, commercial antibody MC-6050 only detected the SNAP-25_{206} uncleaved substrate, and failed to recognize the SNAP-25_{197} cleavage product (Table 8). Even more surprisingly, commercial antibody MC-6050 only detected the SNAP-25_{206} uncleaved substrate, and failed to recognize the SNAP-25_{197} cleavage product, even though the manufacturer advertises that this antibody selectively detects the

SNAP-25_{197} cleavage product (Table 8). Thus, this analysis indicates that while 1D3B8, 2C9B10, 2E2A6, 3C1A5, and 3C3E2 exhibit suitable selectivity for the SNAP-25_{197} cleavage product, 1G10A12 and 2F11B6 do not. In addition, commercial antibodies SMI-81, MC-6050 and MC-6053 all are unsuitable for the immuno-based methods disclosed in the present application because all failed to selectivity detect the SNAP-25_{197} cleavage product.

[0172] For immunocytochemistry analysis, binding specificity was determined by analyzing the ability of α-SNAP-25 antibody-containing ascites to detect the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product by immunostaining. *See e.g.*, Ester Fernandez-Salas et al., *Plasma Membrane Localization Signals in the Light Chain of Botulinum Neurotoxin*, Proc. Natl. Acad. Sci., U.S.A. 101(9): 3208-3213 (2004). A suitable density of PC12 cells were plated, grown, and transfected with either a transfection solution lacking the pQBI-25/GFP-BoNT/A-LC expression construct (untransfected cells) or a transfection solution containing the pQBI-25/GFP-BoNT/A-LC expression construct (transfected cells) as described above. The cells were washed in 1 x PBS and fixed in 5 mL of PAF at room temperature for 30 minutes. Fixed cells were washed in phosphate buffered saline, incubated in 5 mL of 0.5% Triton® X-100 (polyethylene glycol octylphenol ether) in 1 x PBS, washed in 1 x PBS, and permeabilized in 5 mL of methanol at -20 °C for six minutes. Permeabilized cells were blocked in 5 mL of 100 mM glycine at room temperature for 30 minutes, washed in 1 x PBS, and blocked in 5 mL of 0.5% BSA in 1 x PBS at room temperature for 30 minutes. Blocked cells were washed in 1 x PBS and incubated at room temperature for two hours in 0.5% BSA in 1 x PBS containing a 1:10 dilution of an ascites from a clonal hybridoma cell line being tested. Primary antibody probed cells were washed three times for 5 minutes each time in 1 x PBS. Washed cells were incubated at room temperature for 2 hours in 1 x PBS containing a 1:200 dilution of goat polyclonal anti-mouse immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to ALEXA® FLUOR 568 (Invitrogen Inc., Carlsbad, CA) as a secondary antibody. Secondary antibody-probed cells were washed three times for 5 minutes each time in 1 x PBS. Washed cells were prepared for microscopic examination by mounting in VECTASHIELD® Mounting Media (Vector Laboratories, Burlingame, CA) and coverslipped. Images of signal detection were obtained with a Leica confocal microscope using appropriate laser settings. Table 8 indicates that the α-SNAP-25 antibody-containing ascites that specifically detected the SNAP-25_{197}-cleavage product. The immunocytochemistry analysis indicated that ascites produced from clones 1D3B8, 2C9B10, 2E2A6, 3C1A5, and 3C3E2 synthesize an α-SNAP-25 monoclonal antibody having high binding specificity for the SNAP-25_{197} cleavage product that allows for the preferential recognition of this cleavage product relative to the SNAP-25_{206} uncleaved substrate.

[0173] For immunoprecipitation analysis, binding specificity was determined by analyzing the ability of Protein A (HiTrap™ Protein A HP Columns, GE Healthcare, Amersham, Piscataway, NJ), purified α-SNAP-25 monoclonal antibodies to precipitate the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product. *See e.g.*, Chapter 8 *Storing and Purifying Antibodies*, pp. 309-311, Harlow & Lane, *supra*, 1998a. A suitable density of PC12 cells were plated, grown, and transfected with either a

transfection solution containing a pQBI-25/GFP expression construct (control cells; SEQ ID NO: 53) or a transfection solution containing the pQBI-25/GFP-BoNT/A-LC expression construct (experimental cells) as described above. The pQBI-25/GFP expression construct comprises an expression vector whose promoter elements are functionally linked to a polynucleotide encoding GFP of SEQ ID NO: 54. After an overnight incubation, the cells were washed by aspirating the growth media and rinsing each well with 200 µl 1 x PBS. To harvest the cells, the PBS was aspirated, the cells were lysed by adding an Immunoprecipitation Lysis Buffer comprising 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGDT, 10% glycerol, 1% Triton® X-100 (polyethylene glycol octylphenol ether) and a 1 x COMPLETE™ Protease inhibitor cocktail (Roche Applied Biosciences, Indianapolis, IN) and incubating at 4 °C for one hour. The lysed cells were centrifuged at 3,000 x *g* at 4 °C for 10 minutes to remove cellular debris and the supernatant transferred to a clean tube and diluted to a protein concentration of approximately 1 mg/mL. Approximately 5 µg of purified monoclonal antibody was added to 0.5 mL of diluted supernatant and incubated at 4 °C for two hours. After primary antibody incubation, approximately 50 µl of immobilized Protein G (Pierce Biotechnology, Rockford, IL) was added to the diluted supernatant and incubated at 4 °C for one hour. The incubated supernatant was washed three times for 30 minutes each time by adding 0.5 mL of Immunoprecipitation Lysis Buffer, centrifuging at 300 x *g* at 4 °C for one minute to pellet the immobilized Protein G, and decanting the supernatant. After washing, the pellet was resuspended in 30 µl of 1 x SDS Loading Buffer and the sample was heated to 95 °C for 5 minutes. To detect for the presence of both the uncleaved SNAP-25_{206} substrate and the cleaved SNAP-25_{197} product, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that the primary antibody used was a 1:1,000 dilution of the α-SNAP-25 polyclonal antibody serum (see Example IV) and the secondary antibody used was a 1:20,000 of rabbit α-IgG Horseradish Peroxidase (Pierce Biotechnology, Rockford, IL). Table 8 indicates the α-SNAP-25 antibody-containing ascites that specifically pulled down the SNAP-25_{197}-cleavage product by immunoprecipitation analysis. The immunoprecipitation analysis indicated that ascites produced from clones 2E2A6 and 3C1A5 synthesize an α-SNAP-25 monoclonal antibody having high binding specificity for the SNAP-25_{197} cleavage product that allows for the preferential recognition of this cleavage product relative to the SNAP-25_{206} uncleaved substrate.

Table 8. Analysis of Clone Ascites Containing α-SNAP-25 Monoclonal Antibody

Clone	Cell-Based Assay		Immunocytochemistry		Immunoprecipitation	
	SNAP-25_{197}	SNAP-25_{206}	SNAP-25_{197}	SNAP-25_{206}	SNAP-25_{197}	SNAP-25_{206}
1D3B8	++	–	++	-	Not Tested	Not Tested
1G10A12	++	++	Not Tested	Not Tested	Not Tested	Not Tested
2C9B10	++	–	++	-	Not Tested	Not Tested
2E2A6	++	–	++	-	++	–
2F11B6	+	+	+	+	Not Tested	Not Tested
3C1A5	++	–	++	–	++	–
3C3E2	+	–	Not Tested	Not Tested	Not Tested	Not Tested
MC-6050	–	+	Not Tested	Not Tested	Not Tested	Not Tested
MC-6053	–	+	Not Tested	Not Tested	Not Tested	Not Tested

SMI-81	-/+	++	Not Tested	Not Tested	Not Tested	Not Tested

5. Evaluation of binding affinity of α-SNAP-25 monoclonal antibodies.

[0175] To determine the binding affinity of an α-SNAP-25 monoclonal antibody showing high binding specificity for either the SNAP-25_{197} cleavage product or the SNAP-25_{206} uncleaved substrate, binding affinity assays were performed on a BIAcore 3000 instrument using carboxymethyl dextran (CM5) sensor chips (BIAcore, Inc., Piscataway, NJ). Runs were conducted at 25 °C with HBS-EP buffer comprising 10 mM HEPES (pH 7.4), 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant P20 at a flow rate of 10 µl/min. SNAP-25 peptides comprising amino acids 134-197 of SEQ ID NO: 5 (SNAP-$25_{134-197}$) or amino acids 134-206 of SEQ ID NO: 5 (SNAP-$25_{134-206}$) were covalently attached to the surface of the CM5 sensor chips using standard amine coupling. Briefly, the CM5 chips were activated by a 7 minute injection of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccimide; the SNAP-25 peptides were then injected in 10 mM sodium acetate (pH 4.0) for 20 min at a flow rate of 10 µL/min; and unreacted succimide esters were blocked by a 7-min injection of 1 M ethanolamine hydrochloride, pH 8.5. The immobilized amount of SNAP-$25_{134-197}$ or SNAP-$25_{134-206}$ on the chip was reflected by a 100-150 increase in response units (about 0.10-0.15 ng/mm^2). Antibody samples comprising either ascites or purified monoclonal antibodies produced from clones 1D3B8, 2C9B10, 2E2A6, 3C1A5, and 3C3E2, as well as, commercially available α-SNAP-25 antibodies were passed over the surface of the CM5 chips allowing an association time of 10 min and a dissociation time of 20 min. The surfaces were regenerated between runs by a 1 minute injection of 10 mM glycine-HCl (pH 2.5) at a flow rate of 15 µL/min. Sensorgram curves were fitted to a 1:1 kinetic binding model with the BIAevaluation 3.0 software.

[0176] The results indicate that both 2E2A6 and 3C1A5 were highly specific for cleaved SNAP-25_{197} product over SNAP-25 uncleaved substrate (Table 9). When compared to the binding affinities of MC-6050 and MC-6053, 1D3B6 had an approximately 10-fold higher equilibrium disassociation constant for the SNAP-25 cleavage product relative to these commercial antibodies (Table 9). Interestingly, 2E2A6 had only a slightly lower equilibrium disassociation constant for the SNAP-25 cleavage product relative to these commercial antibodies (0.405 nM versus 0.497 and 0.508)(Table 9). As neither of these commercial α-SNAP-25 antibodies selectively recognized the SNAP-25 cleavage product (Table 8), an equilibrium disassociation constant lower than about 0.5 nM appears, in part, critical to achieve such selectivity. Similarly, when compared to the binding affinities of MC-6050 and MC-6053, 2E2A6 had an about at least one-fold slower off rate/dissociation constant (6.74×10^{-5} versus 8.82×10^{-4} s^{-1} and 1.18×10^{-3} s^{-1}) (Table 9). This further suggests that an off rate/dissociation constant lower than about 8.82×10^{-4} appears, in part, critical to achieve selective binding for the SNAP-25 cleavage product. This result is consistent with 1D3B8, which had an off rate/dissociation constant of 5.78×10^{-5} s^{-1} (Table 9).

Table 9. Analysis of Binding Affinity α-SNAP-25 Monoclonal Antibodies		
SPR Parameter	**1D3B8**	**2E2A6***

	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$ [a]	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$ [b]
Ka (M^{-1} s^{-1})	1.06×10^{6}	—	1.70×10^{6} (1.66×10^{5})	— (—)
Kd (s^{-1})	5.78×10^{-5}	—	1.53×10^{-4} (6.74×10^{-5})	— (—)
KD (nM)	0.050	—	0.090 (0.405)	— (—)
SPR Parameter	**3C1A5**		**2C9B10**	
	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$ [c]	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$ [d]
Ka (M^{-1} s^{-1})	2.17×10^{5}	—	1.15×10^{4}	—
Kd (s^{-1})	2.88×10^{-4}	—	3.11×10^{-4}	—
KD (nM)	1.33	—	27.1	—
SPR Parameter	**MC-6050**		**MC-6053**	
	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$	$SNAP\text{-}25_{197}$	$SNAP\text{-}25_{206}$
Ka (M^{-1} s^{-1})	1.78×10^{6}	3.06×10^{2}	2.32×10^{6}	1.06×10^{2}
Kd (s^{-1})	8.82×10^{-4}	6.07×10^{-3}	1.18×10^{-3}	2.56×10^{-5}
KD (nM)	0.497	19,800	0.508	240

* Two independent runs were conducted for this antibody with two different chips.

[a] No binding was observed when up to 125 nM of α-SNAP-25 monoclonal antibody 1D3B8 was passed over the surface of the CM5 sensor chip after a 10 minute association time.

[b] No binding was observed when up to 10 μM of α-SNAP-25 monoclonal antibody 2E2A6 was passed over the surface of the CM5 sensor chip after a 10 minute association time.

[c] No binding was observed when up to 100 nM of α-SNAP-25 monoclonal antibody 3C1A5 was passed over the surface of the CM5 sensor chip after a 10 minute association time.

[d] No binding was observed when up to 100 nM of α-SNAP-25 monoclonal antibody 2C9B10 was passed over the surface of the CM5 sensor chip after a 10 minute association time.

6. Sequencing of the epitope from isolated α-SNAP-25 monoclonal antibodies.

[0177] To determine the epitope of an isolated α-SNAP-25 monoclonal antibody that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, the polynucleotide molecule encoding the variable heavy (V_H) and variable light (V_L) chains of the α-SNAP-25 monoclonal antibody produced by hybridomas 1D3B8, 2C9B10, 2E2A6, 3C1A5 and 3C3E2 were sequenced. mRNA was extracted and purified from each hybridoma using standard protocols and reversed transcribed into cDNA using either an oligo dT anti-sense primer or a gene-specific (murine IgG1 CH and kappa CL) anti-sense primer. Specific murine and human constant domain primers were used to amplify the cDNA by PCR after cDNA production to determine the isotype of the antibody. Degenerate V_H and V_L primers were used to amplify the variable domains from the cDNA. For 5'RACE, a homopolymeric dCTP tail was added to the 3' end of the cDNA. The heavy and light chains were then amplified with an oligo dG sense primer and a gene specific (CH/KC) anti-sense primer. PCR products included the sequence of the signal peptide, variable domains and constant domains up to the anti-sense primer. The PCR products were gel purified to remove small fragments, and cloned into a blunt or TA

vector for sequencing. Five independent clones for each chain were sequenced and alignments of V_H and VL chains and consensus sequences were determined (Table 10). Methods used to determine the V_H and V_L amino acid sequences are described in, *e.g.*, Roger A. Sabbadini, et al., *Novel Bioactive Lipid Derivatives and Methods of Making and Using Same*, U.S. Patent Publication 2007/0281320; and Peter Amersdorfer, et al., *Molecular Characterization of Murine Humoral Immune Response to Botulinum Neurotoxin Type A Binding Domain as Assessed by Using Phage Antibody Libraries*, 65(9) Infect. Immun. 3743-3752, each of which is hereby incorporated by reference in its entirety. In addition, commercial services are available to sequence the variable heavy (V_H) and variable light (V_L) chains of an antibody and identify the CDR regions, see, *e.g.*, Fusion Antibodies Ltd., Northern Ireland.

[0178] The polynucleotide sequence comprising the V_H and V_L chains of the α-SNAP-25 monoclonal antibody produced by the hybridomas disclosed in the present specification is as follows: 1D3B8 V_H (SEQ ID NO: 71), 2C9B10 V_H (SEQ ID NO: 73), 2E2A6 V_H (SEQ ID NO: 75), 3C1A5 V_H variant 1 (SEQ ID NO: 77), 3C1A5 V_H variant 2 (SEQ ID NO: 79), 3C3E2 V_H (SEQ ID NO: 81); 1D3B8 V_L (SEQ ID NO: 83), 2C9B10 V_L (SEQ ID NO: 85), 2E2A6 V_L (SEQ ID NO: 87), 3C1A5 V_L (SEQ ID NO: 89), and 3C3E2 V_L (SEQ ID NO: 91). The amino acid sequence comprising the V_H and V_L chains of the α-SNAP-25 monoclonal antibody produced by the hybridomas disclosed in the present specification is as follows: 1D3B8 V_H (SEQ ID NO: 72), 2C9B10 V_H (SEQ ID NO: 74), 2E2A6 V_H (SEQ ID NO: 76), 3C1A5 V_H variant 1 (SEQ ID NO: 78), 3C1A5 V_H variant 2 (SEQ ID NO: 80), 3C3E2 V_H (SEQ ID NO: 82); 1D3B8 V_L (SEQ ID NO: 84), 2C9B10 V_L (SEQ ID NO: 86), 2E2A6 V_L (SEQ ID NO: 88), 3C1A5 V_L (SEQ ID NO: 90), and 3C3E2 V_L (SEQ ID NO: 92). The amino acid sequences comprising the V_H and V_L CDR domains of the α-SNAP-25 monoclonal antibody produced by the hybridomas 1D3B8, 2C9B10, 2E2A6, 3C1A5, and 3C3E2 are given in Table 10.

Table 10. CDR Sequences of V_H and V_L domains from α-SNAP-25 Monoclonal Antibodies

CDR	Sequence	Identified In	SEQ ID NO:
V_H CDR 1	TFTDHSIH	2E2A6 2C9B10 3C1A5 variant 2	93
V_H CDR 1	TFTNYVIH	3C1A5 variant 1 3C3E2	94
V_H CDR 1	IFTDHALH	1D3B8	95
V_H CDR 2	YIFPGNGNIEYNDKFKG	2E2A6	96
V_H CDR 2	YLFPGNGNFEYNEKFKG	2C9B10 3C1A5 variant 2	97
V_H CDR 2	YINPYNDGSKYNEKFKG	3C1A5 variant 1 3C3E2	98
V_H CDR 2	YIFPGNGNIEYNEKFKG	1D3B8	99
V_H CDR 3	KRMGY	2E2A6 3C1A5 variant 2	100
V_H CDR 3	KKMDY	2C9B10 1D3B8	101
V_H CDR 3	ARHLANTYYYFDY	3C1A5 variant 1 3C3E2	102

V_L CDR 1	RSSQSIVHSNGNTYLE	1D3B8	103
V_L CDR 1	RTTENIYSYFV	2C9B10	104
V_L CDR 1	RASKSVSTSGYSYMH	2E2A6	105
V_L CDR 1	KASQDIKSYLS	3C1A5	106
V_L CDR 1	RASQNIGNYLH	3C3E2	107
V_L CDR 2	KVSNRFS	1D3B8	108
V_L CDR 2	NAKSLAE	2C9B10	109
V_L CDR 2	LVSNLES	2E2A6	110
V_L CDR 2	YATSLAD	3C1A5	111
V_L CDR 2	YASQSIS	3C3E2	112
V_L CDR 3	FQGSHVPPT	1D3B8	113
V_L CDR 3	QHHYGTPYT	2C9B10	114
V_L CDR 3	QHIRELTRS	2E2A6	115
V_L CDR 3	LQHGESPFT	3C1A5	116
V_L CDR 3	QQSDTWPLT	3C3E2	117

[0179] Non-limiting examples of amino acid sequences comprising V_H CDR domain variants of the α-SNAP-25 monoclonal antibody produced by the hybridomas disclosed in the present specification include V_H CDR1 variant SEQ ID NO: 118 for 1D3B8; V_H CDR1 variant SEQ ID NO: 119 for 2C9B10, 2E2A6 and 3C1A5 V_H variant 2; V_H CDR1 variant SEQ ID NO: 120 for 3C1A5 V_H variant 1 and 3C3E2; V_H CDR2 variant SEQ ID NO: 121 for 1D3B8 and 2E2A6; V_H CDR2 variant SEQ ID NO: 122 for 2C9B10 and 3C1A5 V_H variant 2; V_H CDR2 variant SEQ ID NO: 123 for 3C1A5 V_H variant 1, and 3C3E2; V_H CDR3 variant MDY for 1D3B8 and 2C9B10; V_H CDR3 variant MGY for 2E2A6 and 3C1A5 V_H variant 2; and V_H CDR3 variant SEQ ID NO: 124 for 3C1A5 V_H variant 1 and 3C3E2. Non-limiting examples of amino acid sequences comprising V_L CDR domain variants of the α-SNAP-25 monoclonal antibody produced by the hybridomas disclosed in the present specification include V_L CDR1 variant SEQ ID NO: 125 for 1D3B8; V_L CDR1 variant SEQ ID NO: 126 for 2C9B10; V_L CDR1 variant SEQ ID NO: 127 for 2E2A6; V_L CDR1 variant SEQ ID NO: 128 for 3C1A5; V_L CDR1 variant SEQ ID NO: 129 for 3C3E2; V_L CDR2 variant KVS for 1D3B8; V_L CDR2 variant NAK for 2C9B10; V_L CDR2 variant LVS for 2E2A6; V_L CDR2 variant YAT for 3C1A5; and V_L CDR2 variant YAS for 3C3E2.

Example IV

Development of α-SNAP-25 Polyclonal Antibodies that Selectively Bind a SNAP-25 Epitope Having a Free Carboxyl-terminus at the P_1 Residue of the BoNT/A Cleavage Site Scissile Bond

[0180] The following example illustrates how to make α-SNAP-25 polyclonal antibodies that can selectively bind to a SNAP-25 epitope having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

[0181] To develop α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, the 10-

residue peptide CGGGRIDEANQ (SEQ ID NO: 46) was designed as a SNAP-25 cleavage product antigen. This peptide comprising a N-terminal Cysteine residue for conjugation to KLH, a G-spacer flexible spacer (GGG) linked to amino acids 191-197 of human SNAP-25 (SEQ ID NO: 5) and has a carboxylated C-terminal glutamine. Blast searches revealed that this peptide has high homology only to SNAP-25 and almost no possible cross-reactivity with other proteins in neuronal cells. The sequence was also carefully scrutinized by utilizing computer algorithms to determine hydropathy index, protein surface probability, regions of flexibility, and favorable secondary structure, followed by proper orientation and presentation of the chosen peptide sequence. The peptide was synthesized and conjugated to Keyhole Limpet Hemocyanin (KLH) to increase immunogenicity. Before the animals were immunized, naïve rabbits were first screened against cell lysates from candidate cell lines in a Western blot in order to identify animals that had no immunoreactivity to the proteins present in the cell lysates. Two pre-screened rabbits were immunized with this peptide, and after three immunizations in about eight weeks, the rabbits were bled for testing. The blood was allowed to clot by incubating at 4 °C for 60 minutes. The clotted blood was centrifuged at 10,000x g at 4 °C for 10 minutes to pellet the cellular debris. The resulting serum sample was dispensed into 50 µL aliquots and stored at -20 °C until needed.

[0182] A similar strategy based on other SNAP-25 antigens disclosed in the present specification is used to develop α-SNAP-25 polyclonal antibodies that bind an epitope comprising a carboxyl-terminus at the P1 residue from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product. For example, the SNAP-25 antigen of SEQ ID NO: 47 can be conjugated to KLH instead of the SNAP-25 antigen of SEQ ID NO: 46. As another example, the amino acids 191-197 of human SNAP-25 from the SNAP-25 antigen of SEQ ID NO: 38 can be replaced with SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 147, or SEQ ID NO: 148.

2. Screening for the presence of α-SNAP-25 polyclonal antibodies.

[0183] To determine the presence of α-SNAP-25 polyclonal antibodies that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, comparative ELISA and cell-based cleavage assays were performed using the extracted rabbit serum as described in Example III. The serum from both rabbits contained α-SNAP-25 polyclonal antibodies that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond. The α-SNAP-25 rabbit polyclonal antibodies were designated as NTP 22 and NTP 23.

3. Purification of α-SNAP-25 polyclonal antibodies.

[0184] To purify α-SNAP-25 polyclonal antibodies that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, NTP 22 and NTP 23

antibodies from rabbit serum were purified using affinity columns containing the SNAP-25 antigen of SEQ ID NO: 46.

4. Evaluation of binding specificity of α-SNAP-25 polyclonal antibodies.

[0185] To evaluate binding specificity of an α-SNAP-25 polyclonal antibody that can selectively bind to a SNAP-25 antigen having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond, purified NTP 22 and NTP 23 α-SNAP-25 polyclonal antibodies were used to detect cleavage product using the cell-based activity assay, immunnocytochemistry and immunoprecipitation as described in Example III. The cell-based cleavage assay, immunocytochemistry analysis and immunoprecipitation analysis all indicated that NTP 22 and NTP 23 α-SNAP-25 polyclonal antibodies did not cross-react with uncleaved SNAP-25. Thus both NTP 22 and NTP 23 have high binding specificity for the SNAP-25_{197} cleavage product that allows for the preferential recognition of this cleavage product relative to the SNAP-25_{206} uncleaved substrate. Affinity for the antigens can be determined using SPR in the BiAcore as described in Example III.

Example V
Component and Condition Preparation for a Sandwich ELISA

[0186] The following example illustrates how to identify and prepare the components and conditions necessary to perform a sandwich ELISA useful for conducting immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond.

1. Preparation of cell lysates from cells treated with BoNT/A.

[0187] To obtain a BoNT/A treated cell lysate for analysis, a suitable density of cells from a stock culture of Neuro-2a was seeded into a T175 flask containing 50 mL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 2 to 3 days). As a control, a suitable density of cells from a stock culture of Neuro-2a was seeded into a T175 flask containing 50 mL of an appropriate growth medium (Table 1). These undifferentiated control cells were grown in a 37 °C incubator under 5% carbon dioxide until 50% confluence was reached (approximately 18 hours). The media from both differentiated and undifferentiated control cultures was aspirated from each well and replaced with fresh media containing either 0 (untreated sample) or 10 nM of a BoNT/A complex. After an overnight incubation, the cells were washed and the cells harvested by lysing in freshly prepared

Triton X-100 Lysis Buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100) at 4°C for 30 minutes with constant agitation. Lysed cells were centrifuged at 4000 rpm for 20 min at 4°C to eliminate debris using a bench-top centrifuge. The protein concentrations of cell lysates were measured by Bradford assay.

2. Preparation and identification of sandwich ELISA components.

[0188] To identify an appropriate capture antibody-detection antibody pair an ECL sandwich ELISA analysis was conducted on twenty-six different combinations of capture and detection antibody pairs comprising eleven different α-SNAP-25 capture antibodies and seven different α-SNAP-25 detection antibodies (Table 12). The α-SNAP-25 antibodies used were 2E2A6 and 3C1A5 α-SNAP-25 mouse monoclonal antibodies disclosed in the present specification, SMI-81, MC-6050, and MC-6053 α-SNAP-25 mouse monoclonal antibodies disclosed in the present specification, NTP 23 α-SNAP-25 rabbit polyclonal antibodies disclosed in the present specification, S9684 α-SNAP-25 rabbit polyclonal antibodies (Sigma, St. Louis, MO), H-50 α-SNAP-25 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), C-18 α-SNAP-25 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), N-19 α-SNAP-25 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and SP12 α-SNAP-25 mouse polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

[0189] To prepare the capture antibody solution, the α-SNAP-25 monoclonal antibodies contained in the ascites from hybridoma cell lines 2E2A6 and 3C1A5 as well as the α-SNAP-25 MC-6050 and MC-6053 monoclonal antibodies were purified using a standard Protein A purification protocol. All other α-SNAP-25 antibodies were purchased as purified antibodies.

[0190] To prepare the detection antibody solution, the appropriate α-SNAP-25 antibody was conjugated to Ruthenium(II)-tris-bipyridine-(4-methysulfonate) NHS ester labeling reagent (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). The conjugation reaction was performed by adding 30 µL of distilled water reconstituted MSD SULFO-TAG™ stock solution to 200 µL of 2 mg/mL α-SNAP-25 polyclonal antibodies and incubating the reaction at room temperature for 2 hours in the dark. The labeled antibodies were purified using a standard spin column protocol and the protein concentration determined using a standard colorimetric protein assay. The absorbance of the α-SNAP-25 antibody/MSD SULFO-TAG™ conjugate was measured at 455 nm using a spectrophotometer to determine the concentration in moles per liter. The detection antibody solution was stored at 4 °C until needed.

[0191] To prepare the solid phase support comprising the capture antibody that is specific for a SNAP-25 cleavage product, approximately 5 µL of the appropriate α-SNAP-25 monoclonal antibody solution (20 µg/mL in 1 x PBS) is added to each well of a 96-well MSD High Bind plate and the solution is allowed to

air dry in a biological safety cabinet for 2-3 hours in order to liquid evaporate the solution. The capture antibody-bound wells were then blocked by adding 150 µL of Blocking Buffer comprising 2% Amersham Blocking Reagent (GE Life Sciences, Piscataway, NJ) and 10% goat serum (VWR, West Chester, PA) at room temperature for 2 hours. Blocked plates were sealed and stored at 4 °C until needed.

[0192] To detect the presence of a cleaved SNAP-25 cleavage product by ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated from the wells, 25 µL of a lysate from cells treated with BoNT/A, as described above, was added to each well and the plates were incubated at 4 °C for overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µl of 5 µg/mL detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature at room temperature for 1 hour with shaking. After detection antibody incubation, the wells were washed three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 150 µL of 1 x Read Buffer (Meso Scale Discovery, Gaithersburg, MD) was added to each well and the plates were read using a SECTOR™ Imager 6000 Image Reader (Meso Scale Discovery, Gaithersburg, MD). A ratio was calculated by dividing the signal obtained at the 10 nM dose for each antibody-pair by the signal obtained at the 0 nM dose for each antibody-pair (Table 12). These results indicated that among the twenty-six different combinations of antibody pairs tested, only three antibody pairs had signal-to-noise ratios above 10:1 for the higher dose tested: Pair No. 1 (2E2A6 mouse mAb and S9684 rabbit pAb), Pair No. 4 (3C1A5 mouse mAb and S9684 rabbit pAb), and Pair No. 18 (S9684 rabbit pAb and 2E2A6 mouse mAb). Antibody Pair 1 was chosen for further assay development.

Table 12. Screening of α-SNAP-25 Antibody Combinations

Antibody Pair No.	Capture Antibody	Detection Antibody	Detection SNAP-25 cleavage product	Detection SNAP-25 uncleaved substrate	Signal/Noise Ratio (10 nM/0 nM)
1	2E2A6 mouse mAb	S9684 rabbit pAb	Yes	No	26.6:1
2	2E2A6 mouse mAb	N-19 goat pAb	Yes	No	7.3:1
3	2E2A6 mouse mAb	H-50 rabbit pAb	Yes	No	0.9:1
4	3C1A5 mouse mAb	S9684 rabbit pAb	Yes	No	12.1:1
5	3C1A5 mouse mAb	N-19 goat pAb	Yes	No	1.9:1
6	3C1A5 mouse mAb	H-50 rabbit pAb	Yes	No	0.9:1
7	C-18 goat pAb	S9684 rabbit pAb	No	No	0.8:1
8	C-18 goat pAb	N-19 goat pAb	No	No	0.9:1
9	C-18 goat pAb	H-50 rabbit pAb	No	No	0.9:1
10	H-50 rabbit pAb	2E2A6 mouse mAb	Yes	No	0.9:1
11	H-50 rabbit pAb	C-18 goat pAb	No	No	1.0:1
12	N-19 goat pAb	2E2A6 mouse mAb	Yes	No	0.9:1
13	N-19 goat pAb	C-18 goat pAb	No	No	1.1:1
14	NTP 23 rabbit pAb	N-19 goat pAb	Yes	No	1.2:1

15	NTP 23 rabbit pAb	C-18 goat pAb	No	No	1.1:1
16	NTP 23 rabbit pAb	SP12 mouse pAb	Yes	No	1.3:1
17	NTP 23 rabbit pAb	H-50 rabbit pAb	Yes	No	1.1:1
18	S9684 rabbit pAb	2E2A6 mouse mAb	Yes	No	21.3:1
19	S9684 rabbit pAb	C-18 goat pAb	No	No	0.7:1
20	S9684 rabbit pAb	SMI-81mouse mAb	Yes	Yes	1.2:1
21	SMI-81 mouse mAb	S9684 rabbit pAb	Yes	Yes	1.1:1
22	SMI-81 mouse mAb	N-19 goat pAb	Yes	Yes	1.0:1
23	SMI-81 mouse mAb	C-18 goat pAb	No	No	0.8:1
24	SP12 mouse pAb	C-18 goat pAb	No	No	1.0:1
25	MC-6050 mouse mAb	S9684 rabbit pAb	Yes	Yes	5.0:1
26	MC-6053 mouse mAb	S9684 rabbit pAb	Yes	Yes	7.1:1

3. Optimization of cell differentiation conditions.

[0193] To determine the optimal differentiation condition for a cell line comprising cells susceptible to BoNT/A intoxication when using a sandwich ELISA detection system, both various cell culture media and length of differentiation time were tested.

[0194] To determine an optimal differentiation medium, a suitable density of cells from a SiMa cell line was plated into the wells of Collagen IV coated 24-well cell culture plates containing 1 mL of one of the following medias and differentiation supplements: 1) RPMI 1640, 10% fetal bovine serum, 1% Penicillin-Streptomycin, 2 mM L-Glutamine, and 25 µg/mL GT1b); 2) RPMI-1640, 1 x B27 supplement, 1 x N2 supplement, and 25 µg/mL GT1b; 3) Minimum Essential Medium, 1 x B27 supplement, 1 x N2 supplement, and 25 µg/mL GT1b; and 4) RPMI-1640, 10% BSA, 1 x N2 supplement, 1 x NGF supplement, and 25 µg/mL GT1b. Cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.2 pM, 2 pM, or 20 pM of a BoNT/A complex. After an overnight treatment, the cells were washed, incubated for an additional two days without toxin to allow for the cleavage of the SNAP-25 substrate, and harvested as described above in Section 1. The protein concentrations of cell lysates were measured by Bradford assay. Detection of the presence of cleaved SNAP-25 product by ECL sandwich ELISA analysis was performed as described above using Antibody Pair 1. As discussed in Example I, undifferentiated cells did not take up toxin as effectively as differentiated cells. The most effective differentiation medium for increasing BoNT/A uptake and consequently SNAP-25 cleavage medium 3 (MEM+N2+B27), followed by medium 2 (RPMI-1640+N2+B27), and medium 4 (RPMI-1640 +N2+NGF+BSA) (FIG. 3). Cells cultured in medium 2 resulted in more cleavage of the SNAP-25 as compared to the other media.

[0195] To determine an optimal differentiation time, a suitable density of cells from a SiMa cell line was plated into the wells of poly-D-lysine coated 96-well cell culture plates containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b. Cells were plated at four different days to obtain a differentiation time course testing 6 hrs, 24 h, 48 hrs, and 72 hrs, and were incubated in a 37 °C incubator under 5% carbon dioxide The media was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.1 pM, 0.2 pM, 0.4 pM, 0.8 pM, 1.6 pM, 3.1 pM, 6.25 pM, 12.5 pM, or 25 pM of a BoNT/A complex. After an overnight treatment, the cells were washed, incubated for an additional two days without toxin to allow for the cleavage of the SNAP-25 substrate, and harvested as described above in Section 1. After harvesting, the protein concentrations of cell lysates and detection of the presence of cleaved SNAP-25 product by ECL sandwich ELISA analysis were performed as described above. The raw data obtained from the ECL imager was then transferred to SigmaPlot v. 9.0 and a 4-parameter logistics fit was used to define the dose-response curves. There were no constraints used for the 4-parameter logistic function when plotting the data. Graphical reports were generated using the following analysis: R2 (correlation coefficient), a (Max for data set), b (hillslope), and X0 ± SE (EC_{50} value ± standard error). The results indicated that EC_{50} values of less than 2 pM could be achieved with cells differentiated for 48-72 hrs (FIG. 4). The finding that differentiated cells could be used between 48 hrs to 72 hrs of differentiation, with no significant changes on the performance of the cells, highlights the robustness of the assay. Although differentiation time periods less than 48 hrs may not be suitable for picomolar testing of formulated product, these lesser differentiation times are sensitive enough for bulk drug substance testing.

4.* Optimization of *BoNT/A treatment time.

[0196] To determine the optimal length of time cells form a cell line need to be treated with a BoNT/A, various lengths of BoNT/A treatment times were tested. A suitable density of cells from a SiMa cell line was plated into the wells of poly-D-lysine coated 96-well cell culture plates containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b. Cells were plated and incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.1 pM, 0.2 pM, 0.4 pM, 0.8 pM, 1.6 pM, 3.1 pM, 6.3 pM, 12.5 pM, or 25 pM of a BoNT/A complex in RPMI 1640 growth medium in triplicate to generate a full dose-response. Five different BoNT/A treatment length regimens were performed: 1) a 6 hrs BoNT/A treatment, removal and washing of cells, an incubation of cells for 18 hr without BoNT/A, and harvesting of cells as described above in Section 1; 2) a 24 hrs BoNT/A treatment, removal and washing of cells, and harvesting of cells as described above in Section 1; 3) a 24 hrs BoNT/A treatment, removal and washing of cells, an incubation of cells for 24 hr without BoNT/A, and harvesting of cells as described

above in Section 1; 4) a 24 hrs BoNT/A incubation, removal and washing of cells, an incubation of cells for 48 hr without BoNT/A, and harvesting of cells as described above in Section 1; and 5) a 24 hrs BoNT/A incubation, removal and washing of cells, an incubation of cells for 72 hr without BoNT/A, and harvesting of cells as described above in Section 1. After harvesting, the protein concentrations of cell lysates, detection of SNAP-25 cleavage product by ECL sandwich ELISA performed, and the EC_{50} calculated as described above. The results indicate that EC_{50} values of less than 2 pM could be achieved with any of the BoNT/A treatments tested (FIG. 5). Interestingly, the 24 hrs + 24 hrs, 24 hrs + 48 hrs, and 24 hrs + 73 hrs BoNT/A treatment regimes generated essentially the same EC_{50} values, 1.0 pM, 1.1, pM and 0.9 pM respectively. The EC_{50} values generated for the 6 hrs + 18 hrs and 24 hrs + 0 hrs BoNT/A treatment regimes were 1.7 pM and 1.6 pM respectively. Although the amount of signal obtained was lower, these results indicate that BoNT/A treatment times between 6 hrs to 24 hrs plus one day to three days post-treatment incubation can be used to generate an EC_{50} that is adequate for detecting BoNT/A activity and give flexibility in the assay's overall time course.

5. Sensitivity of immuno-based method of detecting BoNT/A activity.

[0197] To evaluate the sensitivity of the immuno-based methods of detecting BoNT/A activity disclosed in the present specification, the timing of BoNT/A uptake by cells susceptible to BoNT/A intoxication was determined. A suitable density of cells from a SiMa cell line was plated into the wells of poly-D-lysine coated 96-well cell culture plates containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 20 µg/mL GT1b. Cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well, replaced with fresh media containing 1 nM of a BoNT/A complex, and the BoNT/A treated cells were incubated at six different time points of 0 min (neurotoxin added and then immediately removed), 5 min, 10 min, 20 min, 30 min, and 60 min. A negative control of media with no BoNT/A (0 nM) was used. After incubation, the cells were washed and harvested as described above in Section 1. After harvesting, the protein concentrations of cell lysates, detection of SNAP-25 cleavage product by ECL sandwich ELISA performed, and the EC_{50} calculated as described above. The results indicated that uptake of BoNT/A by the cells took less than one minute before producing significant amounts of SNAP-25 cleavage product over background (FIG. 6).

6. Specificity of immuno-based method of detecting BoNT/A activity.

[0198] To evaluate the specificity of the immuno-based methods of detecting BoNT/A activity disclosed in the present specification, the capacity of cells susceptible to BoNT/A intoxication to accurately distinguish BoNT/A to the exclusion of partially inactivated BoNT/A was determined. A suitable density of cells from a SiMa cell line was plated into the wells of poly-D-lysine coated 96-well cell culture plates

containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b. Cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well and replaced with fresh media containing either 1) 0 (untreated sample), 0.03 pM, 0.1 pM, 0.31 pM, 0.93 pM, 2.78 pM, 8.33 pM, and 25 pM, of a BoNT/A complex; 2) 0, 0.14 nM, 0.41 nM, 1.23 nM, 3.7 nM, 11.11 nM, 33.33 nM, and 100 nM of an inactive BoNT/A (iBoNT/A); or 3) 0, 0.14 nM, 0.41 nM, 1.23 nM, 3.7 nM, 11.11 nM, 33.33 nM, and 100 nM of an LH_N/A fragment. The iBoNT/A contains a mutation in the zinc binding domain of the light chain that completely inactivates the metalloprotease activity of the neurotoxin, see, *e.g.*, Liqing Zhou, et al., *Expression and Purification of the Light Chain of Botulinum Neurotoxin A: A Single Mutation Abolishes its Cleavage of SNAP-25 and Neurotoxicity after Reconstitution with the Heavy Chain*, Biochemistry 34: 15175-15181 (1995), which is hereby incorporated by reference in its entirety. The LH_N/A fragment lacks the binding domain, but contains an intact translocation domain and light chain, see, *e.g.*, Clifford C. Shone, et al., *Recombinant Toxin Fragments*, U.S. Patent 6,461,617, which is hereby incorporated by reference in its entirety. After 24 hrs treatment, the cells were washed, incubated for an additional two days without toxin to allow for the cleavage of SNAP-25 substrate, and harvested as described above in Section 1. After harvesting, the protein concentrations of cell lysates, detection of SNAP-25 cleavage product by ECL sandwich ELISA performed, and the EC_{50} calculated as described above. The results indicate that the binding affinity of cells for iBoNT/A and LH_N/A (EC_{50} > 100 nM) are at least 60,000 lower than the binding affinity for BoNT/A (EC_{50}= 1.6 pM) (FIG. 7). No SNAP-25 cleavage product was detected in cells treated with iBoNT/A at all concentrations tested. Although a low amount of SNAP-25 cleavage product was detected in cells treated with the highest dose of the LH_N/A fragment, this activity is due to non-specific uptake of this fragment due to the activity of the translocation domain. Thus, the results indicate that the immuno-based methods of detecting BoNT/A activity disclosed in the present specification can measure all the steps involved in the intoxication process whereby a BoNT/A proteolytically cleaves a SNAP-25 substrate and encompasses the binding of a BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the BoNT/A light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a SNAP-25.

Example VI

Immuno-Based method of Detecting BoNT/A Activity Using ECL Sandwich ELISA

[0199] The following example illustrates immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond using ECL sandwich ELISA.

[0200] To prepare a lysate from cells treated with a BoNT/A, a suitable density of cells from an established cell line was plated into the wells of 96-well tissue culture plates containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b (see Examples I and II). These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media from the differentiated cells was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.03 pM, 0.1 pM, 0.3 pM, 0.9 pM, 2.8 pM, 8.3 pM, and 25 pM of a BoNT/A complex. After a 24 hr treatment, the cells were washed, and incubated for an additional two days without toxin. To cells were harvested as described in Example V.

[0201] To prepare the α-SNAP-25 capture antibody solution, the α-SNAP-25 monoclonal antibody contained in the ascites from hybridoma cell line 2E2A6 was purified using a standard Protein A purification protocol To prepare the α-SNAP-25 detection antibody solution, α-SNAP-25 rabbit polyclonal antibody S9684 (Sigma, St. Louis, MO) was conjugated to Ruthenium(II)-tris-bipyridine-(4-methysulfonate) NHS ester labeling reagent (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). The conjugation reaction, purification of labeled α-SNAP-25 antibody, concentration determination and storage were as described in Example V.

[0202] To prepare the solid phase support comprising the capture antibody that is specific for a SNAP-25 cleaved product, approximately 5 µL of α-SNAP-25 monoclonal antibody 2E2A6 solution (20 µg/mL in 1 x PBS) was added to each well of a 96-well MSD High Bind plate and the solution is allowed to air dry in a biological safety cabinet for 2-3 hours in order to liquid evaporate the solution. The capture antibody-bound wells were then blocked and used directly to detect BoNT/A activity.

[0203] To detect the presence of a cleaved SNAP-25 product by ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated from the wells, 25 µL of a lysate from cells treated with BoNT/A was added to each well and the plates were incubated at 4 °C for overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µl of 5 µg/mL detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature at room temperature for 1 hour with shaking. After detection antibody incubation, the wells were washed three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 150 µL of 1 x Read Buffer (Meso Scale Discovery, Gaithersburg, MD) was added to each well and the plates were read using a SECTOR™ Imager 6000 Image Reader (Meso Scale Discovery, Gaithersburg, MD). The collected data was

analyzed and the EC_{50} calculated as described in Example V. A representative result is shown in FIG. 8. These results indicated that on average 1.0 pM of BoNT/A at the EC_{50} was detected (a range of about 0.3 pM to about 2.0 pM) with a signal-to-noise ratio for the lower asymptote of about 15:1 to about 20:1 and a signal-to-noise ratio for the upper asymptote of about 20:1 to about 500:1.

Example VII

Immuno-Based method of Detecting BoNT/A Activity Using CL Sandwich ELISA

[0204] The following example illustrates immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond by CL sandwich ELISA.

[0205] Lysate from cells treated with a BoNT/A and the α-SNAP-25 capture antibody solution were prepared as described in Example VI.

[0206] To prepare the α-SNAP-25 detection antibody solution, α-SNAP-25 polyclonal antibody S9684 (Sigma, St. Louis, MO) was conjugated to Horseradish peroxidase (HRP) according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). The conjugation reaction was performed by adding to 500 µL of 1 mg/mL α-SNAP-25 polyclonal antibodies to a vial containing lyophilized activated peroxidase, mixing the components, and then adding 10 µL of sodium cyanoborohydride. This reaction mixture was incubated at room temperature for 1 hour in a fume hood. After quenching the reaction, the labeled antibodies were purified using a standard spin column protocol and the protein concentration determined using a standard colorimetric protein assay. The absorbance of the α-SNAP-25 polyclonal antibody/HRP conjugate was measured at 455 nm using a spectrophotometer to determine the concentration in moles per liter. The α-SNAP-25 detection antibody solution was stored at 4 °C until needed.

[0207] To prepare the solid phase support comprising the α-SNAP-25 capture antibody that is specific for the SNAP-25 cleaved product, approximately 100 µL of α-SNAP-25 monoclonal antibody 2E2A6 solution (1 mg/mL in 1 x PBS) was added to each well of a 96-well Greiner white plate and the plates were incubated at 4 °C overnight, and then any excess antibody solution was discarded. The capture antibody-bound wells were then blocked by adding 150 µl of Blocking Buffer comprising 2% Amersham Blocking Reagent (GE Life Sciences, Piscataway, NJ) and 10% goat serum (VWR, West Chester, PA) at room temperature for 1 hour. The blocking buffer was discarded and the plates were blotted dry on paper towels by inverting and tapping. The capture antibody-bound wells were then blocked and used directly to detect BoNT/A activity.

[0208] To detect the presence of a cleaved SNAP-25 product by CL sandwich ELISA analysis, 50 µL of a lysate from cells treated with BoNT/A was added to each well, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 500 rpm at 4 °C for 2-4 hours to overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µl 1 x PBS, 0.05 % TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 100 µL of 1 mg/mL α-SNAP-25 polyclonal antibody/HRP detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) was added to each well, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 650 rpm at room temperature for 1 hour. After detection antibody incubation, the wells were washed three times with 200 µl 1 x PBS, 0.05% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 100 µl of SuperSignal ELISA Pico 1:1 mixture (Pierce Biotechnology, Inc., Rockford, IL) was added to each well and the plates were read using a luminometer (Molecular Devices, Sunnyvale, CA) at 395 nm. The collected data was analyzed and the EC_{50} calculated as described in Example V. These results indicated that on average 1.0 pM of BoNT/A at the EC_{50} was detected (a range of about 0.3 pM to about 2.0 pM) with a signal-to-noise ratio for the lower asymptote of about 15:1 to about 20:1 and a signal-to-noise ratio for the upper asymptote of about 20:1 to about 500:1.

Example VIII

Immuno-Based method of Detecting BoNT/A Activity Using Multiplex ECL Sandwich ELISA

[0209] The following example illustrates multiplex immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 cleavage product and a second antibody pair for a different protein.

1. Preparation and identification of capture antibody-detection antibody pair for a second protein.

[0210] To obtain an untreated cell lysate for analysis, a suitable density of cells from a stock culture of SiMa cells were seeded into a T175 flask containing 40 mL of a growth medium containing 1X RPMI 1640, 10% FBS, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES, 1 mM sodium pyruvate, and 100 U/100 µg of pennicillin-streptomycin. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells were approximately 70-90% confluent. The cells were washed and harvested by lysing in freshly prepared Triton X-100 Lysis Buffer (20 mM Tris pH 7.5, 150 mM sodium chloride, 0.001M EDTA, 1 mM EGTA, and 1% Triton-X-100) at 4°C for approximately 30 minutes with constant agitation. Lysed cells were centrifuged at approximately 3300-3330 x g for 40 minutes at 8°C to eliminate debris using a bench-top centrifuge.

[0211] To identify an appropriate capture antibody-detection antibody pair for a second protein, an ECL sandwich ELISA analysis was conducted on 21 different combinations of capture and detection antibody

pairs comprising of five different proteins (Table 13). The antibodies used were α-Syntaxin 1A-HPC mouse monoclonal antibody S0664 (Sigma, St. Louis, MO), α-GAPDH mouse monoclonal antibody MAB374 (Chemicon, Temecula, CA), α-Syntaxin 1 rabbit polyclonal antibody S1172-1 (Sigma, St. Louis, MO), α-GAPDH rabbit polyclonal antibody 2275-PC-1 (R & D Systems, Minneapolis, MN), α-Syntaxin 2 rabbit polyclonal antibody S5687 (Sigma, St. Louis, MO), α-human syntaxin 2 mouse monoclonal antibody MAB2936 (R & D Systems, Minneapolis, MN), α-mouse syntaxin 2 goat polyclonal antibody AF2568 (Sigma, St. Louis, MO), α-Syntaxin 2 rabbit polyclonal antibody AB5596 (Sigma, St. Louis, MO), α-Syntaxin 1 rabbit polyclonal antibody S1172-2 (Sigma, St. Louis, MO), α-h, m, r actin sheep polyclonal antibody AF4000 (R & D Systems, Minneapolis, MN), α-beta actin mouse monoclonal antibody A1978 (Sigma, St. Louis, MO), α-beta mouse polyclonal antibody actin A2228 (Sigma, St. Louis, MO), mouse α-GAPDH mouse monoclonal antibody G8795 (Sigma, St. Louis, MO), α-GAPDH rabbit polyclonal antibody G9595 (Sigma, St. Louis, MO).

[0212] To prepare the second protein capture antibody solution, the monoclonal antibodies were purchased as purified antibodies. To prepare the second protein detection antibody solution, the appropriate antibody was conjugated to Ruthenium(II)-tris-bipyridine-(4-methysulfonate) NHS ester labeling reagent (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). The conjugation reaction was performed by adding 30 µL of distilled water reconstituted MSD SULFO-TAG™ stock solution to 200 µL of 2 mg/mL polyclonal antibodies and incubating the reaction at room temperature for 2 hours in the dark. The labeled antibodies were purified using a standard spin column protocol and the protein concentration determined using a standard colorimetric protein assay. The absorbance of the antibody/MSD SULFO-TAG™ conjugate was measured at 455 nm using a spectrophotometer to determine the concentration in moles per liter. The detection antibody solution was stored at 4 °C until needed.

[0213] To prepare the solid phase support comprising the capture antibody that is specific for a SNAP-25 cleaved product, approximately 5 µL of α-SNAP-25 monoclonal antibody 2E2A6 solution (20 µg/mL in 1 x PBS) was added to each well of a 96-well MSD High Bind plate and the solution is allowed to air dry in a biological safety cabinet for 2-3 hours in order to liquid evaporate the solution, and then the plates were sealed and stored at 4 °C until needed. The dried capture antibody-bound wells were then blocked by adding 150 µL of Blocking Buffer comprising of 3% BSA (Pierce, Rockford, IL) 10% goat serum (Rockland Immunochemicals, Gilbertsville, PA), and Difco 1% skim milk (BD BioSciences Franklin Lakes, NJ) in 0.05% Tween-20 PBS at room temperature for 1-2 hours.

[0214] To detect the presence of protein by ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated from the wells, 25 µL of a lysate from cells treated with BoNT/A, as described above, was added to each well and the plates were incubated at 4 °C for overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µL of 5 µg/mL the

appropriate second protein detection antibody solution, resuspended in the blocking buffer as described above, was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature for about 1 hour with shaking. After detection antibody incubation, the wells were washed three times with 250 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 150 µL of 1 x Read Buffer (Meso Scale Discovery, Gaithersburg, MD) was added to each well and the plates were read using a SECTOR™ Imager 6000 Image Reader (Meso Scale Discovery, Gaithersburg, MD). A ratio was calculated by dividing the signal obtained from the untreated cell lysates for each antibody-pair by the signal obtained for the lysis buffer control (0 nM dose) for each antibody-pair (Table 13). These results indicated that among the twenty-one different combinations of antibody pairs tested, only two antibody pairs had signal-to-noise ratios above 10:1 for the higher dose tested: Pair No. 16 α-GAPDH mouse monoclonal antibody MAB374 and α-GAPDH rabbit polyclonal antibody RDS2275-PC-1; and Pair 21: α-GAPDH mouse monoclonal antibody MAB374 and α-GAPDH rabbit polyclonal antibody G9545. The α-GAPDH mouse monoclonal antibody MAB374 and α-GAPDH rabbit polyclonal antibody G9545 pair was selected as the second protein capture antibody-detection antibody pair for the multiplex ECL sandwich ELISA.

Table 13. Screening of Second Protein Antibody Combinations

Antibody Pair No.	Capture Antibody	Detection Antibody	Detection of Protein	Signal/Noise Ratio (lysate/buffer)
1	α-syntaxin 2 S5687 pAb	α-syntaxin 2 MAB2936 mAb	No	0.92
2	α-syntaxin 2 AF2568 pAb	α-syntaxin 2 AB5596 pAb	No	1.1
3	α-syntaxin 2 AF2568	α-syntaxin 2 S5687 pAb	No	1.11
4	α-syntaxin 2 AF2936 pAb	α-syntaxin 2 AB5596 pAb	Yes	1.63
5	α-syntaxin 2 AF2936 pAb	α-syntaxin 2 S5687 pAb	Yes	1.6
6	α-syntaxin 2 AB5596 pAb	α-syntaxin 2 S5687 pAb	No	0.82
7	α-syntaxin 2 AB5596 pAb	α-syntaxin 2 MAB2936 mAb	No	0.87
8	α-syntaxin 2 MAB2936 mAb	α-syntaxin 2 AB5596 pAb	Yes	1.2
9	α-syntaxin 2 MAB2936 mAb	α-syntaxin 2 S5687 pAb	No	1.07
10	α-syntaxin S0664 mAb	α-syntaxin 1 S1172-1 pAb	Yes	4.23
11	α-syntaxin S0664 mAb	α-syntaxin 1 S1172-2 pAb	No	1.21
12	α-syntaxin 1 S1172-1 pAb	α-syntaxin S0664 mAb	Yes	5.5
13	α-syntaxin 1 S1172-2 pAb	α-syntaxin S0664 mAb	Yes	2.5
14	α-h,m,r actin AF4000 pAb	α-beta actin A1978 mAb	No	1.04
15	α-h,m,r actin AF4000 pAb	α-beta actin A2228 mAb	No	1.08
16	α-GAPDH MAB374 mAb	α-GAPDH 2275-PC-1 pAb	Yes	20.04
17	α-GAPDH MAB374 mAb	α-GAPDH G8795 mAb	No	0.89
18	α-GAPDH 2275-PC-1 pAb	α-GAPDH MAB374 mAb	No	1.08
19	α-GAPDH 2275-PC-1 pAb	α-GAPDH G8795 mAb	Yes	1.27
20	α-GAPDH G8795 mAb	α-GAPDH 2275-PC-1 pAb	Yes	2.74
21	α-GAPDH MAB374 mAb	α-GAPDH G9545 pAb	Yes	≥100

2. Immuno-based method of detecting BoNT/A activity using multiplex ECL sandwich ELISA.

[0215] To obtain a BoNT/A treated cell lysate for analysis, a suitable density of cells from a stock culture of a SiMa cell line were seeded into a poly-D-lysine 96-well plate containing a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.67 U/mL, 2.35 U/mL, 8.23 U/mL, 28.82 U/mL, 101 U/mL, 353 U/mL of a BoNT/A complex. After a 24 hr treatment, the cells were washed, incubated for an additional two days without toxin. The cells were washed, harvested, and processed as described above in Section 1.

[0216] The α-SNAP-25 capture antibody solution and the α-SNAP-25 detection antibody solution, were prepared as described in Example VII. To prepare the α-GAPDH capture antibody solution, the α-GAPDH monoclonal antibody mouse MAB374 (Chemicon, Temecula, CA) was prepared as described in Section 1 above. To prepare the α-GAPDH detection antibody solution, α-GAPDH rabbit polyclonal antibody G9545 (Sigma, St. Louis, MO) was conjugated to Ruthenium(II)-tris-bipyridine-(4-methysulfonate) NHS ester labeling reagent (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). The conjugation reaction, purification of labeled α-SNAP-25 antibody, concentration determination and storage were as described in Section 1 above.

[0217] To prepare the solid phase support comprising the α-SNAP-25capture antibody and the α-GAPDH capture antibody, approximately 2.5 nL of the α-SNAP-25 capture antibody solution (45 µg/mL in 1 x PBS) and 2.5 nL of the α-GAPDH capture antibody solution (120 µg/mL in 1 x PBS) were added to each well of a 96-well MSD High Bind plate in a muliplex format using a robotic system. The solution is allowed to air dry in a biological safety cabinet for at least 2-3 hours in order to liquid evaporate the solution. The capture antibody-bound wells were then blocked and used directly to detect BoNT/A activity and the GAPDH protein.

[0218] To detect the presence of SNAP-25 cleavage product by multiplex ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated from the wells, 25 µL of a lysate from cells treated with BoNT/A, as described above, was added to each well and the plates were incubated at 4 °C for overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µL of 5 µg/mL the α-SNAP-25 detection antibody solution and 25 µL of 5 µg/mL the α-GAPDH detection antibody solution, as described above, was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature for about 1 hour with shaking. After detection antibody incubation, the wells were washed three times with 250 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 150 µL of 1 x Read Buffer (Meso Scale

Discovery, Gaithersburg, MD) was added to each well and the plates were read using a SECTOR™ Imager 6000 Image Reader (Meso Scale Discovery, Gaithersburg, MD). The collected data was analyzed and the relative potency from the normalized data is calculated as described in Example V, except that PLA 2.0 software (Stegmann Systems, GmbH, Germany) was used.

[0219] As a comparison, the detection of SNAP-25 cleavage product was also performed using the singleplex ECL sandwich ELISA as described in Example VI.

[0220] The results indicated that the SNAP-25 data obtained from the singleplex ECL sandwich ELISA, or from the non-normalized SNAP-25 data obtained from the multiplex ECL sandwich ELISA, revealed one outlier sample dose that did not fit into the dose-response curve. However, normalization of the SNAP-25 data against the GAPDH data gave a better curve fit and the potency was closer to the expected value.

Example IX

Immuno-Based method of Detecting BoNT/A Activity Using Multiplex EC Sandwich ELISA

[0221] The following example illustrates multiplex immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 cleavage product and a second antibody pair for a different protein.

[0222] The lysate from cells treated with a BoNT/A was prepared as described in Example VI. The α-SNAP-25 capture antibody solution, the α-SNAP-25 detection antibody solution, and the α-SNAP-25 solid phase support were prepared as described in Example VII.

[0223] To prepare α-GAPDH capture antibody solution, α-GAPDH monoclonal antibody MAB374 (Millipore, Billerica, MA) was purchased as a purified antibody. To prepare the α-GAPDH detection antibody solution, an α-GAPDH polyclonal antibody G9545 (Sigma, St. Louis, MO) was conjugated to Horseradish peroxidase (HRP) according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). The conjugation reaction, concentration determination and storage were as described in Example VII.

[0224] To prepare the solid phase support comprising a second capture antibody specific for the second protein, approximately 100 µL of monoclonal antibody solution comprising 1 µg/mL α-GAPDH monoclonal antibody MAB374 was added to each well of a 96-well Greiner white plate and the plates were incubated at 4 °C overnight, and then any excess antibody solution was discarded. The α-GAPDH capture antibody-bound wells were then blocked by adding 150 µl of Blocking Buffer comprising 2% Amersham Blocking Reagent (GE Life Sciences, Piscataway, NJ) and 10% goat serum (VWR, West Chester, PA) at room temperature for 1 hour. The blocking buffer was discarded and the plates were blotted dry on paper

towels by inverting and tapping. The capture antibody-bound wells were then blocked and used directly to detect BoNT/A activity.

[0225] To detect the presence of a cleaved SNAP-25 product by multiplex CL sandwich ELISA analysis, 50 µL of cell lysates from cells treated with BoNT/A was added to each well of the α-SNAP-25 capture antibody solid phase support and the α-GAPDH capture antibody solid phase support, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 500 rpm at 4 °C for 2-4 hours to overnight. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µl 1 x PBS, 0.05 % TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 100 µL of a detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate), and 1 mg/mL α-SNAP-25 polyclonal antibody/HRP was added to each well of the α-SNAP-25 capture antibody solid phase support, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 650 rpm at room temperature for 1 hour. Similarly, 100 µL of a detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate), and 0.25 mg/mL α-GAPDH G9545 polyclonal antibody/HRP (Sigma Co., St Louis, MO) was added to each well of the α-GAPDH capture antibody solid phase support, the plate was sealed, and the sealed plate was placed on a shaker rotating at 650 rpm at room temperature for 1 hour. After detection antibody incubation, the wells were washed three times with 200 µl 1 x PBS, 0.05% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 100 µl of SuperSignal ELISA Pico 1:1 mixture (Pierce Biotechnology, Inc., Rockford, IL) was added to each well and the plates were read using a luminometer (Molecular Devices, Sunnyvale, CA) at 395 nm. The collected data was analyzed and the EC_{50} calculated as described in Example V. The results indicated that the data points collected for the amounts of α-SNAP-25antibody-antigen complex detected were a better fit after normalization to the amounts of α-GAPDH antibody-antigen complex detected, thereby producing a more accurate reading. These results indicated that on average 1.0 pM of BoNT/A at the EC_{50} was detected (a range of about 0.3 pM to about 2.0 pM) with a signal-to-noise ratio for the lower asymptote of about 15:1 to about 20:1 and a signal-to-noise ratio for the upper asymptote of about 20:1 to about 500:1.

[0226] A similar design can be used for multiplex immuno-based methods of detecting BoNT/A activity by detecting a SNAP-25 cleavage product using a α-SNAP-25 monoclonal antibody specific for a SNAP-25 cleavage product having a carboxyl-terminus at the P_1 residue of the BoNT/A cleavage site scissile bond using ECL sandwich ELISA with the same α-GAPDH antibody pair.

Example X

Immuno-Based Method to Detect Picomolar Amounts of BoNT/A

[0227] The following example illustrates how to perform immuno-based methods of detecting BoNT/A activity that can detect picomolar amounts of the BoNT/A pharmaceutical product, such as, *e.g.*, BOTOX® DYSPORT®/RELOXIN®, PURTOX®, XEOMIN®, NEURONOX®, or BTX-A.

1. Immuno-based method of detecting BoNT/A using ECL sandwich ELISA.

[0228] To prepare a lysate from cells treated with a BoNT/A, approximately 50,000 to 150,000 cells from an established cell line were plated into the wells of 96-well tissue culture poly-D-lysine plates containing 100 µL of a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 µg/mL GT1b (see Examples I and II). These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 2 to 3 days). The media from the differentiated cells was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.03 pM, 0.1 pM, 0.3 pM, 0.9 pM, 2.8 pM, 8.3 pM, or 25 pM of a BoNT/A pharmaceutical product reconstituted in a sodium chloride free solution; or 0 (untreated sample), 0.7 U/mL, 2.1 U/mL, 6.2 U/mL, 18.5 U/mL, 55.6 U/mL, 166.7 U/mL or 500 U/mL of a BoNT/A pharmaceutical product reconstituted in a sodium chloride free medium. Because the BoNT/A pharmaceutical product contains sodium chloride, its addition to the culture medium resulted in a hypertonic media that was detrimental to cell viability. To circumvent the hypertonicity issue, 200 µL of MEM media made without sodium chloride was used to reconstitute the BoNT/A pharmaceutical product giving a final concentration of 25 pM BoNT/A (500 U/mL). The matrix was kept constant for all concentrations along the dose-response curve by adding sodium chloride in the media used to make the dilutions match the amount of excipients present at the highest concentration used (25 pM or 500 U/mL). After a 24 hr treatment, the cells were washed, and incubated for an additional two days without toxin. To harvest the cells, the medium was aspirated, washed with 1 x PBS, and lysed by adding 30 µl of Lysis Buffer comprising 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100 to each well, and the plate incubated on a shaker rotating at 500 rpm for 30 minutes at 4 °C. The plate was centrifuged at 4000 rpm for 20 minutes at 4 °C to pellet cellular debris and the supernatant was transferred to a capture antibody coated 96-well plate to perform the detection step.

[0229] The α-SNAP-25 capture antibody solution, the α-SNAP-25 detection antibody solution, and the solid phase support comprising the capture antibody that is specific for a SNAP-25 cleaved product were prepared as described in Example VI.

[0230] To detect the presence of a cleaved SNAP-25 product by ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated, 25 µL of a lysate from cells treated with BoNT/A was added to each well and the plates were incubated at 4 °C for either 2 hrs or 24 hrs. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µL 1 x PBS,

0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µl of 5 µg/mL α-SNAP-25 detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature for 1 hour with shaking. After α-SNAP-25 detection antibody incubation, the wells were washed three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, the plates were processed, collected data was analyzed, and the EC_{50} calculated as described in Example V. These results indicated that on average 1.0 pM of BoNT/A at the EC_{50} was detected (a range of about 0.3 pM to about 2.0 pM) with a signal-to-noise ratio for the lower asymptote of about 15:1 to about 20:1 and a signal-to-noise ratio for the upper asymptote of about 20:1 to about 500:1 (FIG. 9). This method can also be performed in a multiplex fashion as described in Example VIII.

2. Immuno-based method of detecting BoNT/A using CL sandwich ELISA.

[0231] Lysate from cells treated with a BoNT/A and the α-SNAP-25 capture antibody solution will be prepared as described in Example VI. The α-SNAP-25 detection antibody solution and solid phase support comprising the capture antibody that is specific for a SNAP-25 cleaved product will be prepared as described in Example VII.

[0232] To detect the presence of a cleaved SNAP-25 product by CL sandwich ELISA analysis, 25 µL of a lysate from cells treated with BoNT/A will be added to each well, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 500 rpm at 4 °C for either 2 hrs or 24 hrs. Plate wells will be washed three times by aspirating the cell lysate and rinsing each well three times with 200 µl 1 x PBS, 0.05 % TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 100 µL of 1 mg/mL α-SNAP-25 polyclonal antibody/HRP detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) will be added to each well, the plate was sealed, and the sealed plate was incubated on a shaker rotating at 650 rpm at room temperature for 1 hour. After detection antibody incubation, the wells will be washed three times with 200 µl 1 x PBS, 0.05% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing 100 µl of SuperSignal ELISA Pico 1:1 mixture (Pierce Biotechnology, Inc., Rockford, IL) will be added to each well and the plates will be read using a luminometer (Molecular Devices, Sunnyvale, CA) at 395 nm. The collected data will be analyzed and the EC_{50} will be calculated as described in Example V. This method can also be performed in a multiplex fashion as described in Example VIII.

Example XI

Immuno-Based Method to Detect Neutralizing α-BoNT/A Antibodies

[0233] The following example illustrates how to perform an immuno-based method that can detect the presence of neutralizing α-BoNT/A antibodies.

[0234] BoNT/A, is currently used for a wide range of medical indications including muscle hyperactivity, ophthalmologic, gastrointestinal, urologic, and cosmetic. With repeated long-term treatment of BoNT/A, a patient may develop neutralizing α-BoNT/A antibodies to the toxin leading to immunoresistance. Neutralizing α-BoNT/A antibodies inhibit BoNT/A activity by stopping the toxin's uptake into neuronal cells by binding to the binding domain (H_C) and/or the translocation domain (H_N) of BoNT/A. Some studies have suggested that up to 5-10% of patients repeatedly treated for dystonia with formulations of BoNT/A have immunoresistance due to the development of neutralizing α-BoNT/A antibodies. The established assay to determine the presence of the neutralizing α-BoNT/A antibodies in patient's blood is the mouse protection assay (MPA). Currently, BoNT/A is incubated with a patient's serum prior to injection into mice. The presence of antibodies is manifested by a decreased response to the neurotoxin in the animal. Since the MPA is an in vivo based assay, it would be more cost and time efficient if it was replaced with a cell-based assay.

[0235] To detect the presence or absence of neutralizing α-BoNT/A antibodies, the immuno-based methods of determining BoNT/A activity disclosed in the present specification can be used. One way is to determine the amount of SNAP-25 cleavage product present after treatment with various concentrations of BoNT/A using a Western blot detection method, the other way was to use an ECL sandwich ELISA detection method.

[0236] To prepare a sample comprising neutralizing α-BoNT/A antibodies and a negative control sample known to lack α-BoNT/A neutralizing antibodies, serum was isolated from blood of different individuals. Individuals declining immunizations were referred to as naïve individuals. Individuals accepting immunization were referred to as immunized individuals. The blood was drawn into a serum tube with a clot activator (BD Biosciences, Bedford, MA). Serum was obtained by centrifugation of the blood at 1000xg for 10 minutes at 4°C. The serum of two donors was obtained: one individual was immunized to BoNT/A while the other was not.

[0237] To prepare a lysate from cells treated with a sample comprising BoNT/A, SiMa cells were seeded in a poly-D-lysine 96-well plate and differentiated as described in Example VI. The human serums were serially diluted 1:100 – 1:152,000 by 2.5 fold increments using serum-free media. The BoNT/A was suspended in 0.5 mL SiMa culture media at a concentration of 10 pM. The media containing BoNT/A and α-BoNT/A antibodies were mixed and incubated for 15 min or 1 hr at room temperature. The cells were treated with BoNT/A with human serum for 2 hr followed by a 15 hr incubation in fresh growth media. The cells were also treated for 15 hr with no additional incubation time.

[0238] To detect the presence of a cleaved SNAP-25 product by Western blot analysis, the media was aspirated from each well, the cells suspended in 50 µL of SDS-PAGE loading buffer, and then heated to 95 °C for 5 minutes. An aliquot from each harvested sample was analyzed by Western blot as described

in Example I, except that harvested samples are separated by SDS-PAGE using 12 % 26-well Criterion gels (Bio-Rad Laboratories, Hercules, CA), and the rabbit polyclonal α-SNAP-25_{197} antibody serum was used as the primary antibody (see Example IV). The results indicate that test samples resulted in reduced cleavage of SNAP25 when compared to the negative control sample, demonstrating that the serum from the immunized individual contained neutralizing α-BoNT/A antibodies.

[0239] To detect the presence of a cleaved SNAP-25 product by ECL Sandwich ELISA, the media was removed from each well and the cells were lysed as described in Example V. The α-SNAP-25 capture antibody solution, the α-SNAP-25 detection antibody solution, and the α-SNAP-25 solid phase support were prepared as described in Example VII. Supernatants were transferred to the α-SNAP-25 solid phase support and an ECL sandwich ELISA assay was performed as detailed in Example V. The collected data was analyzed and the EC_{50} calculated as described in Example V, except that the EC_{50} is the serum dilution needed to inhibit the activity of the BoNT/A to ½ its maximum and the ratio of maximal signal ($Signal_{Max}$) to minimum signal ($Signal_{Min}$) was obtained by dividing the SNAP-25 cleavage product signal obtained with the highest dilution of serum by the signal obtained with the lowest serum dilution.

[0240] The results indicate that the presence of neutralizing α-BoNT/A in human serum could be detected. The activity of the BoNT/A complex incubated in serum from the immunized individual decreased as the serum dilution decreased, whereas, the presence of naïve serum had no impact on the assay at every dilution tested. This assay can be performed using a formulated BoNT/A pharmaceutical product, a bulk BoNT/A complex, or a purified neurotoxin.

Example XII

Immuno-Based Method to Detect BoNT/A Activity Using Engineered Cells

[0241] The following example illustrates how to introduce a polynucleotide molecule encoding a BoNT/A receptor into cells from an established cell line to further improve susceptibility to BoNT/A intoxication or improve BoNT/A uptake capacity.

[0242] To introduce an exogenous BoNT/A receptor into cells comprising an established cell line, an expression construct comprising a polynucleotide molecule of SEQ ID NO: 130 encoding the FGFR2 of SEQ ID NO: 59, or a polynucleotide molecule of SEQ ID NO: 139 encoding the FGFR3 of SEQ ID NO: 25, was transfected into cells from an established cell line by a cationic lipid method. A suitable density (about 5 $x10^6$ cells) of cells from an established cell line are plated in a 100 mm tissue culture dish containing 5 mL of complete culture media and grown in a 37 °C incubator under 5% carbon dioxide until the cells reached a density appropriate for transfection. A 3 mL transfection solution is prepared by adding 1.5 mL of OPTI-MEM Reduced Serum Medium containing 60 µL of LipofectAmine 2000 (Invitrogen, Carlsbad, CA) incubated at room temperature for 5 minutes to 1.5 mL of OPTI-MEM Reduced Serum Medium containing 24 µg of an expression construct encoding a FGFR2 or a FGFR3, or a control

expression construct encoding a green fluorescent protein (GFP). This transfection mixture was incubated at room temperature for approximately 30 minutes. The complete media is replaced with the 3 mL transfection solution and the cells are incubated in a 37 °C incubator under 5% carbon dioxide for approximately 8 hours. Transfection media is replaced with 3 mL of fresh complete culture media and the cells are incubated in a 37 °C incubator under 5% carbon dioxide for approximately 24 hours. Media is replaced with 3 mL of fresh complete culture media containing approximately 1mM G418 (Invitrogen, Carlsbad, CA). Cells are incubated in a 37 °C incubator under 5% carbon dioxide for approximately 1 week, the old media is replaced with fresh complete culture media containing 0.5 mM G418. Once antibiotic-resistant colonies are established, resistant clones are replated to new 100 mm culture plates containing fresh complete culture media, supplemented with approximately 0.5 mM G418 until these cells reached a density of 6 to $20x10^5$ cells/mL.

[0243] To determine if the overexpression of BoNT/A receptors improved cell susceptibility to BoNT/A intoxication or improved BoNT/A uptake capacity, a dose-response curve was generated using cells treated with different doses of a BoNT/A complex. To prepare a lysate from cells treated with a BoNT/A, a suitable density of cells from an established transfected cell line was plated into the wells of 96-well tissue culture plates containing 100 µL of an appropriate serum-free medium (Table 5). These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media from the differentiated cells was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.01 nM, 0.04 nM, 0.12 nM, 0.37 nM, 1.1 nM, 3.3 nM, and 10 nM of a BoNT/A complex for cells comprising a SiMa or a PC12 transfected cell line; and 0 (untreated sample), 0.14 nM, 0.40 nM, 1.2 nM, 3.7 nM, 11 nM, 33 nM, and 100 nM of a BoNT/A complex for cells comprising a Neuro-2a transfected cell line. The cells were treated with BoNT/A containing media for 6 hrs followed by incubation with fresh media for 15 hrs and harvested by adding 40 µL of 2 x SDS-PAGE loading buffer and heating the plate to 95 °C for 5 min.

[0244] To detect for the presence of SNAP-25 cleavage product, an aliquot from each harvested sample was analyzed by Western blot as described in Example I, except that harvested samples are separated by SDS-PAGE using 12% 26-well Criterion gels (Bio-Rad Laboratories, Hercules, CA), and the following primary antibodies were used a 1:1,000 dilution of rabbit polyclonal α-SNAP-25 antibody serum (Example IV) (AGN, polyclonal antibody), a 1:500 dilution of α-FGFR2 rabbit polyclonal C-17 (Santa Cruz Biotechnology, Santa Cruz, CA), or a 1:500 dilution of α-FGFR3 rabbit polyclonal C-15 (Santa Cruz Biotechnology, Santa Cruz, CA). The intensity of the protein of interest from each sample was calculated using Image Quant (GE Healthcare, Piscataway, NJ) and the EC_{50} for each of the cells lines was estimated using SigmaPlot software.

[0245] The results indicate that cells transfected with FGFR2 or FGFR3 were more sensitive to BoNT/A than cells transfected with GFP and also showed a higher level of SNAP-25 cleavage (Table 14). The

EC_{50} values for cells over-expressing FGFR2 or FGFR3 were lower than the EC_{50} values exhibited by cells over-expressing GFP, indicating that introduction of FGFR2 or FGFR3 improved cell susceptibility to BoNT/A intoxication or improved BoNT/A uptake capacity.

Table 14. Effects of Introducing Exogenous BoNT/A Receptors on Cell Susceptibilty to BoNT/A Intoxication or BoNT/A Uptake

Cells	Transfected Gene	EC_{50} (nM)	Max Signal
SiMa	GFP	0.0812 ± 0.010	22,733,787
SiMa	FGFR2	0.0459 ± 0.003	26,136,578
SiMa	FGFR3	0.0377 ± 0.006	24,326,271
PC-12	GFP	3.3362 ± 1.881	26,956,063
PC-12	FGFR2	0.3429 ± 0.059	25,376,114
PC-12	FGFR3	0.2634 ± 0.026	24,102,459
Neuro-2a	GFP	61.80 ± 9.710	4,605,974
Neuro-2a	FGFR2	31.59 ± 8.800	23,279,765
Neuro-2a	FGFR3	11.55 ± 5.240	28,347,413

[0246] Detection for the presence of SNAP-25 cleavage product can also be performed using sandwich ELISA as described in Examples VI-X.

What is claimed:

1. A composition comprising a carrier, a flexible linker and a SNAP-25 antigen, wherein the flexible linker comprises at least three amino acids and the SNAP-25 antigen comprises SEQ ID NO: 148.

2. The composition of Claim 2, wherein the flexible linker and the SNAP-25 antigen amino acid sequence is SEQ ID NO: 38.

3. An isolated α-SNAP-25 antibody,

   wherein the isolated α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product;

   wherein the α-SNAP-25 antibody has an association rate constant for an epitope not comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product of less than 1×10^{1} M^{-1} s^{-1}; and

   wherein the α-SNAP-25 antibody has an equilibrium disassociation constant for the epitope of less than 0.450 nM.

4. The isolated α-SNAP-25 antibody of Claim 3, wherein the epitope is SEQ ID NO: 32.

5. An isolated α-SNAP-25 antibody comprising,

   a. a heavy chain variable region comprising SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 80, or SEQ ID NO: 82; and

   b. a light chain variable region comprising SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, or SEQ ID NO: 92.

6. An isolated α-SNAP-25 antibody comprising,

   a. a heavy chain variable region comprising SEQ ID NO: 93, SEQ ID NO: 121, or SEQ ID NO: 100; and

   b. a light chain variable region comprising SEQ ID NO: 105, SEQ ID NO: 110, or SEQ ID NO: 115.

7. An isolated α-SNAP-25 antibody comprising at least a V_H CDR3 of SEQ ID NO: 100, a V_H CDR3 of SEQ ID NO: 101, or a V_H CDR3 of SEQ ID NO: 102.

8. A method of detecting BoNT/A activity, the method comprising the steps of:

   a. treating a cell from an established cell line with a sample comprising a BoNT/A, wherein the cell from an established cell line is susceptible to BoNT/A intoxication by about 500 pM or less of a BoNT/A;

   b. isolating from the treated cell a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond;

   c. contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support,

      wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, the α-SNAP-25 antibody has an association rate constant for an epitope from a SNAP-25 not comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product of less than 1×10^1 M^{-1} s^{-1}; and the α-SNAP-25 antibody has an equilibrium disassociation constant for the epitope of less than 0.450 nM;

   d. detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product having a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond;

   wherein detection by the antibody-antigen complex is indicative of BoNT/A activity.

9. The method of Claim 8, wherein the SNAP-25 cleavage product is $SNAP\text{-}25_{197}$.

10. The method of Claim 8, wherein the presence of an antibody-antigen complex is detected using a sandwich ELISA.

11. The method of Claim 8, wherein the method has a signal-to-noise ratio at the lower asymptote of at least 3:1 and a signal-to-noise ratio at the upper asymptote of at least 10:1.

12. The method of Claim 8, wherein the sample comprises at most 100 pM of a BoNT/A

13. The method of Claim 8, wherein the cell from an established cell line is susceptible to BoNT/A intoxication by about 100 pM or less of a BoNT/A.

14. The method of Claim 8, wherein the method is performed in a singleplex fashion or a multiplex fashion.

15. A method of determining BoNT/A immunoresistance in a mammal comprising the steps of:

a. adding a BoNT/A to a test sample obtained from a mammal being tested for the presence or absence of α-BoNT/A neutralizing antibodies;

b. treating a cell from an established cell line with the test sample, wherein the cell from an established cell line is susceptible to BoNT/A intoxication;

c. isolating from the treated cells a SNAP-25 component comprising a SNAP-25 cleavage product having a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond;

d. contacting the SNAP-25 component with an α-SNAP-25 antibody linked to a solid phase support, wherein the α-SNAP-25 antibody;

wherein the α-SNAP-25 antibody binds an eptiope comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product, the α-SNAP-25 antibody has an association rate constant for an epitope from a SNAP-25 not comprising a carboxyl-terminus glutamine of the BoNT/A cleavage site scissile bond from a SNAP-25 cleavage product of less than 1×10^{1} M^{-1} s^{-1}; and the α-SNAP-25 antibody has an equilibrium disassociation constant for the epitope of less than 0.450 nM;

e. detecting the presence of an antibody-antigen complex comprising the α-SNAP-25 antibody and the SNAP-25 cleavage product having a carboxyl-terminus glutamine from the BoNT/A cleavage site scissile bond;

f. repeating steps b-e with a negative control sample instead of a test sample, the negative control sample comprising a BoNT/A and a serum known not to contain α-BoNT/A neutralizing antibodies; and

g. comparing the amount of antibody-antigen complex detected in step e to the amount of antibody-antigen complex detected in step f,

wherein detection of a lower amount of antibody-antigen complex detected in step e relative to the amount of antibody-antigen complex detected in step f is indicative of the presence of α-BoNT/A neutralizing antibodies.

SEQUENCE LISTING

<110> Fernandez-Salas, Ester
Wang, Joanne
Garay, Patton E.
Wong, Lina S.
Hodges, D. Dianne
Aoki, Kei Roger

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Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Ser Asp Leu Ile Gln
                485                 490                 495
Gln Tyr Tyr Leu Thr Phe Asp Phe Asp Asn Glu Pro Glu Asn Ile Ser
            500                 505                 510
Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu Glu Pro Met Pro
        515                 520                 525
Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu Leu Asp Lys Tyr
    530                 535                 540
Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu His Gly Asp Ser
545                 550                 555                 560
Arg Ile Ile Leu Thr Asn Ser Ala Glu Glu Ala Leu Leu Lys Pro Asn
                565                 570                 575
Val Ala Tyr Thr Phe Phe Ser Ser Lys Tyr Val Lys Lys Ile Asn Lys
            580                 585                 590
Ala Val Glu Ala Val Ile Phe Leu Ser Trp Ala Glu Glu Leu Val Tyr
        595                 600                 605
Asp Phe Thr Asp Glu Thr Asn Glu Val Thr Thr Met Asp Lys Ile Ala
    610                 615                 620
Asp Ile Thr Ile Ile Val Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly
625                 630                 635                 640
Asn Met Val Ser Lys Gly Glu Phe Val Glu Ala Ile Leu Phe Thr Gly
                645                 650                 655
Val Val Ala Leu Leu Glu Phe Ile Pro Glu Tyr Ser Leu Pro Val Phe
            660                 665                 670
Gly Thr Phe Ala Ile Val Ser Tyr Ile Ala Asn Lys Val Leu Thr Val
        675                 680                 685
Gln Thr Ile Asn Asn Ala Leu Ser Lys Arg Asn Glu Lys Trp Asp Glu
    690                 695                 700
Val Tyr Lys Tyr Thr Val Thr Asn Trp Leu Ala Lys Val Asn Thr Gln
705                 710                 715                 720
Ile Asp Leu Ile Arg Glu Lys Met Lys Lys Ala Leu Glu Asn Gln Ala
                725                 730                 735
Glu Ala Thr Arg Ala Ile Ile Asn Tyr Gln Tyr Asn Gln Tyr Thr Glu
            740                 745                 750
Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp Leu Ser Ser Lys
        755                 760                 765
Leu Asn Arg Ser Ile Asn Arg Ala Met Ile Asn Ile Asn Lys Phe Leu
    770                 775                 780
Asp Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met Ile Pro Tyr Ala
785                 790                 795                 800
Val Lys Arg Leu Lys Asp Phe Asp Ala Ser Val Arg Asp Val Leu Leu
                805                 810                 815
Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Leu Gln Val Asp Arg
            820                 825                 830
Leu Lys Asp Glu Val Asn Asn Thr Leu Ser Ala Asp Ile Pro Phe Gln
```

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        835                 840                 845
Leu Ser Lys Tyr Val Asn Asp Lys Lys Leu Leu Ser Thr Phe Thr Glu
    850                 855                 860
Tyr Ile Lys Asn Ile Val Asn Thr Ser Ile Leu Ser Ile Val Tyr Lys
865                 870                 875                 880
Lys Asp Asp Leu Ile Asp Leu Ser Arg Tyr Gly Ala Lys Ile Asn Ile
                885                 890                 895
Gly Asp Arg Val Tyr Tyr Asp Ser Ile Asp Lys Asn Gln Ile Lys Leu
            900                 905                 910
Ile Asn Leu Glu Ser Ser Thr Ile Glu Val Ile Leu Lys Asn Ala Ile
        915                 920                 925
Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile Lys
    930                 935                 940
Ile Pro Lys Tyr Phe Ser Lys Ile Asn Leu Asn Asn Glu Tyr Thr Ile
945                 950                 955                 960
Ile Asn Cys Ile Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr
                965                 970                 975
Gly Glu Ile Ile Trp Thr Leu Gln Asp Asn Lys Gln Asn Ile Gln Arg
            980                 985                 990
Val Val Phe Lys Tyr Ser Gln Met Val Asn Ile Ser Asp Tyr Ile Asn
        995                 1000                1005
Arg Trp Met Phe Val Thr Ile Thr Asn Asn Arg Leu Thr Lys Ser Lys
    1010                1015                1020
Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu
1025                1030                1035                1040
Gly Asn Ile His Ala Ser Asn Lys Ile Met Phe Lys Leu Asp Gly Cys
                1045                1050                1055
Arg Asp Pro Arg Arg Tyr Ile Met Ile Lys Tyr Phe Asn Leu Phe Asp
            1060                1065                1070
Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Ser Gln Ser
        1075                1080                1085
Asn Pro Gly Ile Leu Lys Asp Phe Trp Gly Asn Tyr Leu Gln Tyr Asp
    1090                1095                1100
Lys Pro Tyr Tyr Met Leu Asn Leu Phe Asp Pro Asn Lys Tyr Val Asp
1105                1110                1115                1120
Val Asn Asn Ile Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg
                1125                1130                1135
Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Thr Leu Tyr Met
            1140                1145                1150
Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Glu Asp Asn
        1155                1160                1165
Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys Asn
    1170                1175                1180
Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu Lys
1185                1190                1195                1200
Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln Val
                1205                1210                1215
Val Val Met Lys Ser Lys Asp Asp Gln Gly Ile Arg Asn Lys Cys Lys
            1220                1225                1230
Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Val Gly Phe
        1235                1240                1245
His Leu Tyr Asp Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn
    1250                1255                1260
Arg Gln Val Gly Lys Ala Ser Arg Thr Phe Gly Cys Ser Trp Glu Phe
1265                1270                1275                1280
Ile Pro Val Asp Asp Gly Trp Gly Glu Ser Ser Leu
                1285                1290
```

<210> 4
<211> 1296
<212> PRT
<213> Clostridium botulinum

<400> 4

```
Met Pro Leu Val Asn Gln Gln Ile Asn Tyr Tyr Asp Pro Val Asn Gly
 1               5                  10                  15
Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Ala Gly Lys Met Gln Pro
            20                  25                  30
Val Lys Ala Phe Lys Ile His Asn Lys Val Trp Val Ile Pro Glu Arg
        35                  40                  45
Asp Ile Phe Thr Asn Pro Glu Glu Val Asp Leu Asn Pro Pro Pro Glu
    50                  55                  60
Ala Lys Gln Val Pro Ile Ser Tyr Tyr Asp Ser Ala Tyr Leu Ser Thr
65                  70                  75                  80
Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Ile Lys Leu Phe Glu
                85                  90                  95
Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Ile Ser Ile Val
            100                 105                 110
Arg Gly Ile Pro Phe Trp Gly Gly Gly Lys Ile Asp Thr Glu Leu Lys
        115                 120                 125
Val Ile Asp Thr Asn Cys Ile Asn Ile Ile Gln Leu Asp Asp Ser Tyr
    130                 135                 140
Arg Ser Glu Glu Leu Asn Leu Ala Ile Ile Gly Pro Ser Ala Asn Ile
145                 150                 155                 160
Ile Glu Ser Gln Cys Ser Ser Phe Arg Asp Asp Val Leu Asn Leu Thr
                165                 170                 175
Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe
            180                 185                 190
Thr Val Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu
        195                 200                 205
Gly Ala Gly Lys Phe Ala Gln Asp Pro Ala Val Ala Leu Ala His Glu
    210                 215                 220
Leu Ile His Ala Glu His Arg Leu Tyr Gly Ile Ala Ile Asn Thr Asn
225                 230                 235                 240
Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ala Gly Leu
                245                 250                 255
Glu Val Ser Leu Glu Glu Leu Ile Thr Phe Gly Gly Asn Asp Ala Lys
            260                 265                 270
Phe Ile Asp Ser Leu Gln Lys Lys Glu Phe Ser Leu Tyr Tyr Tyr Asn
        275                 280                 285
Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val
    290                 295                 300
Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys
305                 310                 315                 320
Tyr Leu Leu Ser Glu Asp Ala Thr Gly Lys Phe Leu Val Asp Arg Leu
                325                 330                 335
Lys Phe Asp Glu Leu Tyr Lys Leu Leu Thr Glu Ile Tyr Thr Glu Asp
            340                 345                 350
Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn
        355                 360                 365
Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Asp Val Asn Tyr
    370                 375                 380
Thr Ile His Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn
385                 390                 395                 400
Phe Asn Gly Gln Asn Ile Glu Ile Asn Asn Lys Asn Phe Asp Lys Leu
                405                 410                 415
Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg
```

```
            420                 425                 430
Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Glu Gly Tyr Asn Lys
        435                 440                 445
Ala Leu Asn Glu Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe
    450                 455                 460
Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asp Lys Val Glu Glu
465                 470                 475                 480
Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu
                485                 490                 495
Asp Leu Ile Gln Gln Tyr Tyr Leu Asn Phe Asn Phe Asp Asn Glu Pro
            500                 505                 510
Glu Asn Thr Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu
        515                 520                 525
Glu Pro Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu
    530                 535                 540
Leu Asn Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Lys
545                 550                 555                 560
His Ser Asn Ser Arg Ile Ile Leu Thr Asn Ser Ala Lys Glu Ala Leu
                565                 570                 575
Leu Lys Pro Asn Ile Val Tyr Thr Phe Phe Ser Ser Lys Tyr Ile Lys
            580                 585                 590
Ala Ile Asn Lys Ala Val Glu Ala Val Thr Phe Val Asn Trp Ile Glu
        595                 600                 605
Asn Leu Val Tyr Asp Phe Thr Asp Glu Thr Asn Glu Val Ser Thr Met
    610                 615                 620
Asp Lys Ile Ala Asp Ile Thr Ile Val Ile Pro Tyr Ile Gly Pro Ala
625                 630                 635                 640
Leu Asn Ile Gly Asn Met Ile Tyr Lys Gly Glu Phe Val Glu Ala Ile
                645                 650                 655
Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Ile Val Pro Glu Ile Ala
            660                 665                 670
Leu Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Val Ser Asn Lys
        675                 680                 685
Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu
    690                 695                 700
Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Ile
705                 710                 715                 720
Val Asn Thr Gln Ile Asn Leu Ile Arg Glu Lys Met Lys Lys Ala Leu
                725                 730                 735
Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn
            740                 745                 750
Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp
        755                 760                 765
Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Ser Ala Met Ile Asn Ile
    770                 775                 780
Asn Lys Phe Leu Asp Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met
785                 790                 795                 800
Ile Pro Tyr Ala Val Lys Arg Leu Lys Asp Phe Asp Ala Ser Val Arg
                805                 810                 815
Asp Val Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly
            820                 825                 830
Gln Val Asn Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Ala Asp
        835                 840                 845
Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Lys Lys Leu Leu Ser
    850                 855                 860
Thr Phe Thr Glu Tyr Ile Lys Asn Ile Thr Asn Ala Ser Ile Leu Ser
865                 870                 875                 880
Ile Val Tyr Lys Asp Asp Asp Leu Ile Asp Leu Ser Arg Tyr Gly Ala
                885                 890                 895
```

Glu Ile Tyr Asn Gly Asp Lys Val Tyr Tyr Asn Ser Ile Asp Lys Asn
900 905 910
Gln Ile Arg Leu Ile Asn Leu Glu Ser Ser Thr Ile Glu Val Ile Leu
915 920 925
Lys Lys Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser
930 935 940
Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn
945 950 955 960
Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val
965 970 975
Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Phe Gln Asp Thr Gln Glu
980 985 990
Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser
995 1000 1005
Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Ile
1010 1015 1020
Thr Lys Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro
1025 1030 1035 1040
Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Lys Ile Met Phe Lys
1045 1050 1055
Leu Asp Gly Cys Arg Asp Pro His Arg Tyr Ile Val Ile Lys Tyr Phe
1060 1065 1070
Asn Leu Phe Asp Lys Glu Leu Ser Glu Lys Glu Ile Lys Asp Leu Tyr
1075 1080 1085
Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr
1090 1095 1100
Leu Gln Tyr Asp Lys Ser Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn
1105 1110 1115 1120
Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu
1125 1130 1135
Lys Gly Pro Arg Asp Asn Val Met Thr Thr Asn Ile Tyr Leu Asn Ser
1140 1145 1150
Ser Leu Tyr Met Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly
1155 1160 1165
Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val
1170 1175 1180
Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala
1185 1190 1195 1200
Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn
1205 1210 1215
Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr
1220 1225 1230
Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly
1235 1240 1245
Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser
1250 1255 1260
Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys
1265 1270 1275 1280
Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Arg Glu Arg Pro Leu
1285 1290 1295

<210> 5
<211> 206
<212> PRT
<213> Homo sapiens

<400> 5
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg

```
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Asp Arg Val Glu Glu Gly Met
    50                  55                  60
Asn His Ile Asn Gln Asp Met Lys Glu Ala Glu Lys Asn Leu Lys Asp
65                  70                  75                  80
Leu Gly Lys Cys Cys Gly Leu Phe Ile Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205
```

<210> 6
<211> 206
<212> PRT
<213> Homo sapiens

<400> 6

```
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205
```

<210> 7
<211> 206
<212> PRT
<213> Macaca mulatta

<400> 7
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
1 5 10 15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
20 25 30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
35 40 45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
50 55 60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65 70 75 80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
85 90 95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
100 105 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
115 120 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
130 135 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145 150 155 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
165 170 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
180 185 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
195 200 205

<210> 8
<211> 206
<212> PRT
<213> Rattus norvegicus

<400> 8
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
1 5 10 15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
20 25 30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
35 40 45
Met Leu Asp Glu Gln Gly Glu Gln Leu Asp Arg Val Glu Glu Gly Met
50 55 60
Asn His Ile Asn Gln Asp Met Lys Glu Ala Glu Lys Asn Leu Lys Asp
65 70 75 80
Leu Gly Lys Cys Cys Gly Leu Phe Ile Cys Pro Cys Asn Lys Leu Lys
85 90 95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
100 105 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
115 120 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn

```
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205
```

<210> 9
<211> 206
<212> PRT
<213> Rattus norvegicus

<400> 9

```
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205
```

<210> 10
<211> 206
<212> PRT
<213> Mus musculus

<400> 10

```
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
```

```
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205


<210> 11
<211> 206
<212> PRT
<213> Gallus gallus

<400> 11
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile
            180                 185                 190
Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205


<210> 12
<211> 204
<212> PRT
<213> Carassius auratus
```

<400> 12
Met Ala Glu Asp Ala Asp Met Arg Asn Glu Leu Ser Asp Met Gln Gln
1 5 10 15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
20 25 30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
35 40 45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
50 55 60
Asp Gln Ile Asn Lys Asp Met Lys Asp Ala Glu Lys Asn Leu Asn Asp
65 70 75 80
Leu Gly Lys Phe Cys Gly Leu Cys Ser Cys Pro Cys Asn Lys Met Lys
85 90 95
Ser Gly Gly Ser Lys Ala Trp Gly Asn Asn Gln Asp Gly Val Val Ala
100 105 110
Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala Ile Ser
115 120 125
Gly Gly Phe Ile Arg Arg Val Thr Asp Asp Ala Arg Glu Asn Glu Met
130 135 140
Asp Glu Asn Leu Glu Gln Val Gly Gly Ile Ile Gly Asn Leu Arg His
145 150 155 160
Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg Gln Ile
165 170 175
Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile Asp Glu
180 185 190
Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
195 200

<210> 13
<211> 203
<212> PRT
<213> Carassius auratus

<400> 13
Met Ala Asp Glu Ala Asp Met Arg Asn Glu Leu Thr Asp Met Gln Ala
1 5 10 15
Arg Ala Asp Gln Leu Gly Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
20 25 30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
35 40 45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
50 55 60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65 70 75 80
Leu Gly Asn Leu Cys Gly Leu Cys Pro Cys Pro Cys Asn Lys Leu Lys
85 90 95
Gly Gly Gly Gln Ser Trp Gly Asn Asn Gln Asp Gly Val Val Ser Ser
100 105 110
Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala Ile Ser Gly
115 120 125
Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn Glu Met Asp
130 135 140
Glu Asn Leu Glu Gln Val Gly Ser Ile Ile Gly Asn Leu Arg His Met
145 150 155 160
Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg Gln Ile Asp
165 170 175
Arg Ile Met Asp Met Ala Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala
180 185 190

```
Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200


<210> 14
<211> 204
<212> PRT
<213> Danio rerio

<400> 14
Met Ala Glu Asp Ser Asp Met Arg Asn Glu Leu Ala Asp Met Gln Gln
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Asp Gln Ile Asn Lys Asp Met Lys Asp Ala Glu Lys Asn Leu Asn Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Ser Cys Pro Cys Asn Lys Met Lys
                85                  90                  95
Ser Gly Ala Ser Lys Ala Trp Gly Asn Asn Gln Asp Gly Val Val Ala
            100                 105                 110
Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala Ile Ser
        115                 120                 125
Gly Gly Phe Ile Arg Arg Val Thr Asp Asp Ala Arg Glu Asn Glu Met
    130                 135                 140
Asp Glu Asn Leu Glu Gln Val Gly Gly Ile Ile Gly Asn Leu Arg His
145                 150                 155                 160
Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg Gln Ile
                165                 170                 175
Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Thr Arg Ile Asp Glu
            180                 185                 190
Ala Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200


<210> 15
<211> 203
<212> PRT
<213> Danio rerio

<400> 15
Met Ala Asp Glu Ser Asp Met Arg Asn Glu Leu Asn Asp Met Gln Ala
 1               5                  10                  15
Arg Ala Asp Gln Leu Gly Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Asn Leu Cys Gly Leu Cys Pro Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Gly Gly Gly Gln Ser Trp Gly Asn Asn Gln Asp Gly Val Val Ser Ser
            100                 105                 110
Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala Ile Ser Gly
```

```
        115                 120                 125
Gly Phe Ile Arg Arg Val Thr Asn Asp Ala Arg Glu Asn Glu Met Asp
    130                 135                 140
Glu Asn Leu Glu Gln Val Gly Ser Ile Ile Gly Asn Leu Arg His Met
145                 150                 155                 160
Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg Gln Ile Asp
                165                 170                 175
Arg Ile Met Asp Met Ala Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala
            180                 185                 190
Asn Gln Arg Ala Thr Lys Met Leu Gly Ser Gly
        195                 200
```

<210> 16
<211> 210
<212> PRT
<213> Torpedo marmorata

<400> 16

```
Met Glu Asn Ser Val Glu Asn Ser Met Asp Pro Arg Ser Glu Gln Glu
 1               5                  10                  15
Glu Met Gln Arg Cys Ala Asp Gln Ile Thr Asp Glu Ser Leu Glu Ser
            20                  25                  30
Thr Arg Arg Met Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile
        35                  40                  45
Arg Thr Leu Val Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile
    50                  55                  60
Glu Glu Gly Met Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys
65                  70                  75                  80
Asn Leu Ser Asp Leu Gly Lys Cys Cys Gly Leu Cys Ser Cys Pro Cys
                85                  90                  95
Asn Lys Leu Lys Asn Phe Glu Ala Gly Gly Ala Tyr Lys Lys Val Trp
            100                 105                 110
Gly Asn Asn Gln Asp Gly Val Val Ala Ser Gln Pro Ala Arg Val Met
        115                 120                 125
Asp Asp Arg Glu Gln Met Ala Met Ser Gly Gly Tyr Ile Arg Arg Ile
    130                 135                 140
Thr Asp Asp Ala Arg Glu Asn Glu Met Glu Glu Asn Leu Asp Gln Val
145                 150                 155                 160
Gly Ser Ile Ile Gly Asn Leu Arg His Met Ala Leu Asp Met Ser Asn
                165                 170                 175
Glu Ile Gly Ser Gln Asn Ala Gln Ile Asp Arg Ile Val Val Lys Gly
            180                 185                 190
Asp Met Asn Lys Ala Arg Ile Asp Glu Ala Asn Lys His Ala Thr Lys
        195                 200                 205
Met Leu
    210
```

<210> 17
<211> 206
<212> PRT
<213> Xenopus laevis

<400> 17

```
Met Ala Asp Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
```

```
Leu Gln Tyr Val Glu Gly Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Asp Arg Val Glu Glu Gly Met
    50                  55                  60
Asn His Ile Asn Gln Asp Met Lys Glu Ala Glu Lys Asn Leu Lys Asp
65                  70                  75                  80
Leu Gly Lys Cys Cys Gly Leu Phe Ile Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Gly Ala Tyr Asn Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Val Arg Arg Val Thr Asn Asp Ala Arg Glu Thr
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Gly Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Ala Arg Ile
            180                 185                 190
Asp Glu Ala Asn Lys His Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205


<210> 18
<211> 206
<212> PRT
<213> Xenopus laevis

<400> 18
Met Ala Asp Asp Ala Asp Met Arg Asn Glu Leu Glu Glu Met Gln Arg
 1               5                  10                  15
Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
            20                  25                  30
Leu Gln Tyr Val Glu Gly Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
        35                  40                  45
Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
    50                  55                  60
Glu Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65                  70                  75                  80
Leu Gly Lys Phe Cys Gly Leu Cys Val Cys Pro Cys Asn Lys Leu Lys
                85                  90                  95
Ser Ser Asp Ala Tyr Lys Lys Ala Trp Gly Asn Asn Gln Asp Gly Val
            100                 105                 110
Val Ala Ser Gln Pro Ala Arg Val Val Asp Glu Arg Glu Gln Met Ala
        115                 120                 125
Ile Ser Gly Gly Phe Val Arg Arg Val Thr Asn Asp Ala Arg Glu Thr
    130                 135                 140
Glu Met Asp Glu Asn Leu Glu Gln Val Gly Gly Ile Ile Gly Asn Leu
145                 150                 155                 160
Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr Gln Asn Arg
                165                 170                 175
Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser Asn Lys Ala Arg Ile
            180                 185                 190
Asp Glu Ala Asn Lys His Ala Thr Lys Met Leu Gly Ser Gly
        195                 200                 205


<210> 19
```

<211> 212
<212> PRT
<213> Strongylocentrotus purpuratus

<400> 19
Met Glu Asp Gln Asn Asp Met Asn Met Arg Ser Glu Leu Glu Glu Ile
1 5 10 15
Gln Met Gln Ser Asn Met Gln Thr Asp Glu Ser Leu Glu Ser Thr Arg
20 25 30
Arg Met Leu Gln Met Ala Glu Glu Ser Gln Asp Met Gly Ile Lys Thr
35 40 45
Leu Val Met Leu Asp Glu Gln Gly Glu Gln Leu Asp Arg Ile Glu Glu
50 55 60
Gly Met Asp Gln Ile Asn Thr Asp Met Arg Glu Ala Glu Lys Asn Leu
65 70 75 80
Thr Gly Leu Glu Lys Cys Cys Gly Ile Cys Val Cys Pro Trp Lys Lys
85 90 95
Leu Gly Asn Phe Glu Lys Gly Asp Asp Tyr Lys Lys Thr Trp Lys Gly
100 105 110
Asn Asp Asp Gly Lys Val Asn Ser His Gln Pro Met Arg Met Glu Asp
115 120 125
Asp Arg Asp Gly Cys Gly Gly Asn Ala Ser Met Ile Thr Arg Ile Thr
130 135 140
Asn Asp Ala Arg Glu Asp Glu Met Asp Glu Asn Leu Thr Gln Val Ser
145 150 155 160
Ser Ile Val Gly Asn Leu Arg His Met Ala Ile Asp Met Gln Ser Glu
165 170 175
Ile Gly Ala Gln Asn Ser Gln Val Gly Arg Ile Thr Ser Lys Ala Glu
180 185 190
Ser Asn Glu Gly Arg Ile Asn Ser Ala Asp Lys Arg Ala Lys Asn Ile
195 200 205
Leu Arg Asn Lys
210

<210> 20
<211> 212
<212> PRT
<213> Drosophila melanogaster

<400> 20
Met Pro Ala Asp Pro Ser Glu Glu Val Ala Pro Gln Val Pro Lys Thr
1 5 10 15
Glu Leu Glu Glu Leu Gln Ile Asn Ala Gln Gly Val Ala Asp Glu Ser
20 25 30
Leu Glu Ser Thr Arg Arg Met Leu Ala Leu Cys Glu Glu Ser Lys Glu
35 40 45
Ala Gly Ile Arg Thr Leu Val Ala Leu Asp Asp Gln Gly Glu Gln Leu
50 55 60
Asp Arg Ile Glu Glu Gly Met Asp Gln Ile Asn Ala Asp Met Arg Glu
65 70 75 80
Ala Glu Lys Asn Leu Ser Gly Met Glu Lys Cys Cys Gly Ile Cys Val
85 90 95
Leu Pro Cys Asn Lys Ser Gln Ser Phe Lys Glu Asp Asp Gly Thr Trp
100 105 110
Lys Gly Asn Asp Asp Gly Lys Val Val Asn Asn Gln Pro Gln Arg Val
115 120 125
Met Asp Asp Arg Asn Gly Met Met Ala Gln Ala Gly Tyr Ile Gly Arg
130 135 140

```
Ile Thr Asn Asp Ala Arg Glu Asp Glu Met Glu Glu Asn Met Gly Gln
145                 150                 155                 160
Val Asn Thr Met Ile Gly Asn Leu Arg Asn Met Ala Leu Asp Met Gly
                165                 170                 175
Ser Glu Leu Glu Asn Gln Asn Arg Gln Ile Asp Arg Ile Asn Arg Lys
            180                 185                 190
Gly Glu Ser Asn Glu Ala Arg Ile Ala Val Ala Asn Gln Arg Ala His
        195                 200                 205
Gln Leu Leu Lys
    210


<210> 21
<211> 212
<212> PRT
<213> Hirudo medicinalis

<400> 21
Met Ala Lys Asp Ile Lys Pro Lys Pro Ala Asn Gly Arg Asp Ser Pro
 1               5                  10                  15
Thr Asp Leu Gln Glu Ile Gln Leu Gln Met Asn Ala Ile Thr Asp Asp
            20                  25                  30
Ser Leu Glu Ser Thr Arg Arg Met Leu Ala Met Cys Glu Glu Ser Lys
        35                  40                  45
Asp Ala Gly Ile Arg Thr Leu Val Met Leu Asp Glu Gln Gly Glu Gln
    50                  55                  60
Leu Asp Arg Ile Glu Glu Gly Met Asp Gln Ile Asn Gln Asp Met Arg
65                  70                  75                  80
Asp Ala Glu Lys Asn Leu Glu Gly Met Glu Lys Cys Cys Gly Leu Cys
                85                  90                  95
Ile Leu Pro Trp Lys Arg Thr Lys Asn Phe Asp Lys Gly Ala Glu Trp
            100                 105                 110
Asn Lys Gly Asp Glu Gly Lys Val Asn Thr Asp Gly Pro Arg Leu Val
        115                 120                 125
Val Gly Asp Gly Asn Met Gly Pro Ser Gly Gly Phe Ile Thr Lys Ile
    130                 135                 140
Thr Asn Asp Ala Arg Glu Glu Glu Met Glu Gln Asn Met Gly Glu Val
145                 150                 155                 160
Ser Asn Met Ile Ser Asn Leu Arg Asn Met Ala Val Asp Met Gly Ser
                165                 170                 175
Glu Ile Asp Ser Gln Asn Arg Gln Val Asp Arg Ile Asn Asn Lys Met
            180                 185                 190
Thr Ser Asn Gln Leu Arg Ile Ser Asp Ala Asn Lys Arg Ala Ser Lys
        195                 200                 205
Leu Leu Lys Glu
    210


<210> 22
<211> 212
<212> PRT
<213> Loligo pealei

<400> 22
Met Ser Ala Asn Gly Glu Val Glu Val Pro Lys Thr Glu Leu Glu Glu
 1               5                  10                  15
Ile Gln Gln Gln Cys Asn Gln Val Thr Asp Asp Ser Leu Glu Ser Thr
            20                  25                  30
Arg Arg Met Leu Asn Met Cys Glu Glu Ser Lys Glu Ala Gly Ile Arg
```

```
        35                  40                  45
Thr Leu Val Met Leu Asp Glu Gln Gly Glu Gln Leu Asp Arg Ile Glu
    50                  55                  60
Glu Gly Leu Asp Gln Ile Asn Gln Asp Met Lys Asp Ala Glu Lys Asn
65                  70                  75                  80
Leu Glu Gly Met Glu Lys Cys Cys Gly Leu Cys Val Leu Pro Trp Lys
                85                  90                  95
Arg Gly Lys Ser Phe Glu Lys Ser Gly Asp Tyr Ala Asn Thr Trp Lys
            100                 105                 110
Lys Asp Asp Asp Gly Pro Thr Asn Thr Asn Gly Pro Arg Val Thr Val
        115                 120                 125
Gly Asp Gln Asn Gly Met Gly Pro Ser Ser Gly Tyr Val Thr Arg Ile
    130                 135                 140
Thr Asn Asp Ala Arg Glu Asp Asp Met Glu Asn Asn Met Lys Glu Val
145                 150                 155                 160
Ser Ser Met Ile Gly Asn Leu Arg Asn Met Ala Ile Asp Met Gly Asn
                165                 170                 175
Glu Ile Gly Ser Gln Asn Arg Gln Val Asp Arg Ile Gln Gln Lys Ala
            180                 185                 190
Glu Ser Asn Glu Ser Arg Ile Asp Glu Ala Asn Lys Lys Ala Thr Lys
        195                 200                 205
Leu Leu Lys Asn
    210
```

<210> 23
<211> 220
<212> PRT
<213> Lymnaea stagnalis

<400> 23

```
Met Thr Thr Asn Gly Glu Ile Leu Pro Val Gly Glu Glu Glu Glu Glu
 1               5                  10                  15
Glu Leu Gly Glu Asp Ala Leu Leu Arg Lys Gln Ile Asp Cys Asn Thr
            20                  25                  30
Asn Glu Ser Leu Glu Ser Thr Arg Arg Met Leu Ser Leu Cys Glu Glu
        35                  40                  45
Ser Lys Glu Ala Gly Ile Lys Thr Leu Val Met Leu Asp Glu Gln Gly
    50                  55                  60
Glu Gln Leu Asp Arg Ile Glu Glu Gly Met Gly Gln Ile Asn Gln Asp
65                  70                  75                  80
Met Arg Asp Ala Glu Lys Asn Leu Glu Gly Leu Glu Lys Cys Cys Gly
                85                  90                  95
Leu Cys Val Leu Pro Trp Lys Arg Ser Lys Asn Phe Glu Lys Gly Ser
            100                 105                 110
Asp Tyr Asn Lys Thr Trp Lys Ala Ser Glu Asp Gly Lys Ile Asn Thr
        115                 120                 125
Asn Gly Pro Arg Leu Val Val Asp Gln Gly Asn Gly Ser Gly Pro Thr
    130                 135                 140
Gly Gly Tyr Ile Thr Arg Ile Thr Asn Asp Ala Arg Glu Asp Glu Met
145                 150                 155                 160
Glu Gln Asn Ile Gly Glu Val Ala Gly Met Val Ser Asn Leu Arg Asn
                165                 170                 175
Met Ala Val Asp Met Gly Asn Glu Ile Glu Ser Gln Asn Lys Gln Leu
            180                 185                 190
Asp Arg Ile Asn Gln Lys Gly Gly Ser Leu Asn Val Arg Val Asp Glu
        195                 200                 205
Ala Asn Lys Arg Ala Asn Arg Ile Leu Arg Lys Gln
    210                 215                 220
```

<210> 24
<211> 207
<212> PRT
<213> Caenorhabditis elegans

<400> 24
Met Ser Gly Asp Asp Asp Ile Pro Glu Gly Leu Glu Ala Ile Asn Leu
1 5 10 15
Lys Met Asn Ala Thr Thr Asp Asp Ser Leu Glu Ser Thr Arg Arg Met
20 25 30
Leu Ala Leu Cys Glu Glu Ser Lys Glu Ala Gly Ile Lys Thr Leu Val
35 40 45
Met Leu Asp Asp Gln Gly Glu Gln Leu Glu Arg Cys Glu Gly Ala Leu
50 55 60
Asp Thr Ile Asn Gln Asp Met Lys Glu Ala Glu Asp His Leu Lys Gly
65 70 75 80
Met Glu Lys Cys Cys Gly Leu Cys Val Leu Pro Trp Asn Lys Thr Asp
85 90 95
Asp Phe Glu Lys Thr Glu Phe Ala Lys Ala Trp Lys Lys Asp Asp Asp
100 105 110
Gly Gly Val Ile Ser Asp Gln Pro Arg Ile Thr Val Gly Asp Ser Ser
115 120 125
Met Gly Pro Gln Gly Gly Tyr Ile Thr Lys Ile Thr Asn Asp Ala Arg
130 135 140
Glu Asp Glu Met Asp Glu Asn Val Gln Gln Val Ser Thr Met Val Gly
145 150 155 160
Asn Leu Arg Asn Met Ala Ile Asp Met Ser Thr Glu Val Ser Asn Gln
165 170 175
Asn Arg Gln Leu Asp Arg Ile His Asp Lys Ala Gln Ser Asn Glu Val
180 185 190
Arg Val Glu Ser Ala Asn Lys Arg Ala Lys Asn Leu Ile Thr Lys
195 200 205

<210> 25
<211> 808
<212> PRT
<213> Homo sapiens

<400> 25
Met Gly Ala Pro Ala Cys Ala Leu Ala Leu Cys Val Ala Val Ala Ile
1 5 10 15
Val Ala Gly Ala Ser Ser Glu Ser Leu Gly Thr Glu Gln Arg Val Val
20 25 30
Gly Arg Ala Ala Glu Val Pro Gly Pro Glu Pro Gly Gln Gln Glu Gln
35 40 45
Leu Val Phe Gly Ser Gly Asp Ala Val Glu Leu Ser Cys Pro Pro Pro
50 55 60
Gly Gly Gly Pro Met Gly Pro Thr Val Trp Val Lys Asp Gly Thr Gly
65 70 75 80
Leu Val Pro Ser Glu Arg Val Leu Val Gly Pro Gln Arg Leu Gln Val
85 90 95
Leu Asn Ala Ser His Glu Asp Ser Gly Ala Tyr Ser Cys Arg Gln Arg
100 105 110
Leu Thr Gln Arg Val Leu Cys His Phe Ser Val Arg Val Thr Asp Ala
115 120 125
Pro Ser Ser Gly Asp Asp Glu Asp Gly Glu Asp Glu Ala Glu Asp Thr

```
    130                 135                 140
Gly Val Asp Thr Gly Ala Pro Tyr Trp Thr Arg Pro Glu Arg Met Asp
145                 150                 155                 160
Lys Lys Leu Leu Ala Val Pro Ala Ala Asn Thr Val Arg Phe Arg Cys
                165                 170                 175
Pro Ala Ala Gly Asn Pro Thr Pro Ser Ile Ser Trp Leu Lys Asn Gly
            180                 185                 190
Arg Glu Phe Arg Gly Glu His Arg Ile Gly Gly Ile Lys Leu Arg His
        195                 200                 205
Gln Gln Trp Ser Leu Val Met Glu Ser Val Val Pro Ser Asp Arg Gly
    210                 215                 220
Asn Tyr Thr Cys Val Val Glu Asn Lys Phe Gly Ser Ile Arg Gln Thr
225                 230                 235                 240
Tyr Thr Leu Asp Val Leu Glu Arg Ser Pro His Arg Pro Ile Leu Gln
                245                 250                 255
Ala Gly Leu Pro Ala Asn Gln Thr Ala Val Leu Gly Ser Asp Val Glu
            260                 265                 270
Phe His Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile Gln Trp Leu
        275                 280                 285
Lys His Val Glu Val Asn Gly Ser Lys Val Gly Pro Asp Gly Thr Pro
    290                 295                 300
Tyr Val Thr Val Leu Lys Ser Trp Ile Ser Glu Ser Val Glu Ala Asp
305                 310                 315                 320
Val Arg Leu Arg Leu Ala Asn Val Ser Glu Arg Asp Gly Gly Glu Tyr
                325                 330                 335
Leu Cys Arg Ala Thr Asn Phe Ile Gly Val Ala Glu Lys Ala Phe Trp
            340                 345                 350
Leu Ser Val His Gly Pro Arg Ala Ala Glu Glu Glu Leu Val Glu Ala
        355                 360                 365
Asp Glu Ala Gly Ser Val Tyr Ala Gly Ile Leu Ser Tyr Gly Val Gly
    370                 375                 380
Phe Phe Leu Phe Ile Leu Val Val Ala Ala Val Thr Leu Cys Arg Leu
385                 390                 395                 400
Arg Ser Pro Pro Lys Lys Gly Leu Gly Ser Pro Thr Val His Lys Ile
                405                 410                 415
Ser Arg Phe Pro Leu Lys Arg Gln Val Ser Leu Glu Ser Asn Ala Ser
            420                 425                 430
Met Ser Ser Asn Thr Pro Leu Val Arg Ile Ala Arg Leu Ser Ser Gly
        435                 440                 445
Glu Gly Pro Thr Leu Ala Asn Val Ser Glu Leu Glu Leu Pro Ala Asp
    450                 455                 460
Pro Lys Trp Glu Leu Ser Arg Ala Arg Leu Thr Leu Gly Lys Pro Leu
465                 470                 475                 480
Gly Glu Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Ile Gly Ile
                485                 490                 495
Asp Lys Asp Arg Ala Ala Lys Pro Val Thr Val Ala Val Lys Met Leu
            500                 505                 510
Lys Asp Asp Ala Thr Asp Lys Asp Leu Ser Asp Leu Val Ser Glu Met
        515                 520                 525
Glu Met Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn Leu Leu
    530                 535                 540
Gly Ala Cys Thr Gln Gly Gly Pro Leu Tyr Val Leu Val Glu Tyr Ala
545                 550                 555                 560
Ala Lys Gly Asn Leu Arg Glu Phe Leu Arg Ala Arg Arg Pro Pro Gly
                565                 570                 575
Leu Asp Tyr Ser Phe Asp Thr Cys Lys Pro Pro Glu Glu Gln Leu Thr
            580                 585                 590
Phe Lys Asp Leu Val Ser Cys Ala Tyr Gln Val Ala Arg Gly Met Glu
        595                 600                 605
```

```
Tyr Leu Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala Arg Asn
    610                 615                 620
Val Leu Val Thr Glu Asp Asn Val Met Lys Ile Ala Asp Phe Gly Leu
625                 630                 635                 640
Ala Arg Asp Val His Asn Leu Asp Tyr Tyr Lys Lys Thr Thr Asn Gly
                645                 650                 655
Arg Leu Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp Arg Val
            660                 665                 670
Tyr Thr His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu Trp Glu
        675                 680                 685
Ile Phe Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val Glu Glu
    690                 695                 700
Leu Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ala Asn
705                 710                 715                 720
Cys Thr His Asp Leu Tyr Met Ile Met Arg Glu Cys Trp His Ala Ala
                725                 730                 735
Pro Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu Asp Arg
            740                 745                 750
Val Leu Thr Val Thr Ser Thr Asp Glu Tyr Leu Asp Leu Ser Ala Pro
        755                 760                 765
Phe Glu Gln Tyr Ser Pro Gly Gly Gln Asp Thr Pro Ser Ser Ser Ser
    770                 775                 780
Ser Gly Asp Asp Ser Val Phe Ala His Asp Leu Leu Pro Pro Ala Pro
785                 790                 795                 800
Pro Ser Ser Gly Gly Ser Arg Thr
                805
```

<210> 26
<211> 806
<212> PRT
<213> Homo sapiens

<400> 26

```
Met Gly Ala Pro Ala Cys Ala Leu Ala Leu Cys Val Ala Val Ala Ile
 1               5                  10                  15
Val Ala Gly Ala Ser Ser Glu Ser Leu Gly Thr Glu Gln Arg Val Val
            20                  25                  30
Gly Arg Ala Ala Glu Val Pro Gly Pro Glu Pro Gly Gln Gln Glu Gln
        35                  40                  45
Leu Val Phe Gly Ser Gly Asp Ala Val Glu Leu Ser Cys Pro Pro Pro
    50                  55                  60
Gly Gly Gly Pro Met Gly Pro Thr Val Trp Val Lys Asp Gly Thr Gly
65                  70                  75                  80
Leu Val Pro Ser Glu Arg Val Leu Val Gly Pro Gln Arg Leu Gln Val
                85                  90                  95
Leu Asn Ala Ser His Glu Asp Ser Gly Ala Tyr Ser Cys Arg Gln Arg
            100                 105                 110
Leu Thr Gln Arg Val Leu Cys His Phe Ser Val Arg Val Thr Asp Ala
        115                 120                 125
Pro Ser Ser Gly Asp Asp Glu Asp Gly Glu Asp Glu Ala Glu Asp Thr
    130                 135                 140
Gly Val Asp Thr Gly Ala Pro Tyr Trp Thr Arg Pro Glu Arg Met Asp
145                 150                 155                 160
Lys Lys Leu Leu Ala Val Pro Ala Ala Asn Thr Val Arg Phe Arg Cys
                165                 170                 175
Pro Ala Ala Gly Asn Pro Thr Pro Ser Ile Ser Trp Leu Lys Asn Gly
            180                 185                 190
Arg Glu Phe Arg Gly Glu His Arg Ile Gly Gly Ile Lys Leu Arg His
```

```
        195                 200                 205
Gln Gln Trp Ser Leu Val Met Glu Ser Val Val Pro Ser Asp Arg Gly
    210                 215                 220
Asn Tyr Thr Cys Val Val Glu Asn Lys Phe Gly Ser Ile Arg Gln Thr
225                 230                 235                 240
Tyr Thr Leu Asp Val Leu Glu Arg Ser Pro His Arg Pro Ile Leu Gln
                245                 250                 255
Ala Gly Leu Pro Ala Asn Gln Thr Ala Val Leu Gly Ser Asp Val Glu
            260                 265                 270
Phe His Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile Gln Trp Leu
        275                 280                 285
Lys His Val Glu Val Asn Gly Ser Lys Val Gly Pro Asp Gly Thr Pro
    290                 295                 300
Tyr Val Thr Val Leu Lys Thr Ala Gly Ala Asn Thr Thr Asp Lys Glu
305                 310                 315                 320
Leu Glu Val Leu Ser Leu His Asn Val Thr Phe Glu Asp Ala Gly Glu
                325                 330                 335
Tyr Thr Cys Leu Ala Gly Asn Ser Ile Gly Phe Ser His His Ser Ala
            340                 345                 350
Trp Leu Val Val Leu Pro Ala Glu Glu Glu Leu Val Glu Ala Asp Glu
        355                 360                 365
Ala Gly Ser Val Tyr Ala Gly Ile Leu Ser Tyr Gly Val Gly Phe Phe
    370                 375                 380
Leu Phe Ile Leu Val Val Ala Ala Val Thr Leu Cys Arg Leu Arg Ser
385                 390                 395                 400
Pro Pro Lys Lys Gly Leu Gly Ser Pro Thr Val His Lys Ile Ser Arg
                405                 410                 415
Phe Pro Leu Lys Arg Gln Val Ser Leu Glu Ser Asn Ala Ser Met Ser
            420                 425                 430
Ser Asn Thr Pro Leu Val Arg Ile Ala Arg Leu Ser Ser Gly Glu Gly
        435                 440                 445
Pro Thr Leu Ala Asn Val Ser Glu Leu Glu Leu Pro Ala Asp Pro Lys
    450                 455                 460
Trp Glu Leu Ser Arg Ala Arg Leu Thr Leu Gly Lys Pro Leu Gly Glu
465                 470                 475                 480
Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Ile Gly Ile Asp Lys
                485                 490                 495
Asp Arg Ala Ala Lys Pro Val Thr Val Ala Val Lys Met Leu Lys Asp
            500                 505                 510
Asp Ala Thr Asp Lys Asp Leu Ser Asp Leu Val Ser Glu Met Glu Met
        515                 520                 525
Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn Leu Leu Gly Ala
    530                 535                 540
Cys Thr Gln Gly Gly Pro Leu Tyr Val Leu Val Glu Tyr Ala Ala Lys
545                 550                 555                 560
Gly Asn Leu Arg Glu Phe Leu Arg Ala Arg Arg Pro Pro Gly Leu Asp
                565                 570                 575
Tyr Ser Phe Asp Thr Cys Lys Pro Pro Glu Glu Gln Leu Thr Phe Lys
            580                 585                 590
Asp Leu Val Ser Cys Ala Tyr Gln Val Ala Arg Gly Met Glu Tyr Leu
        595                 600                 605
Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala Arg Asn Val Leu
    610                 615                 620
Val Thr Glu Asp Asn Val Met Lys Ile Ala Asp Phe Gly Leu Ala Arg
625                 630                 635                 640
Asp Val His Asn Leu Asp Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu
                645                 650                 655
Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp Arg Val Tyr Thr
            660                 665                 670
```

```
His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu Trp Glu Ile Phe
        675                 680                 685
Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val Glu Glu Leu Phe
    690                 695                 700
Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ala Asn Cys Thr
705                 710                 715                 720
His Asp Leu Tyr Met Ile Met Arg Glu Cys Trp His Ala Ala Pro Ser
                725                 730                 735
Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu Asp Arg Val Leu
            740                 745                 750
Thr Val Thr Ser Thr Asp Glu Tyr Leu Asp Leu Ser Ala Pro Phe Glu
        755                 760                 765
Gln Tyr Ser Pro Gly Gly Gln Asp Thr Pro Ser Ser Ser Ser Ser Gly
    770                 775                 780
Asp Asp Ser Val Phe Ala His Asp Leu Leu Pro Pro Ala Pro Pro Ser
785                 790                 795                 800
Ser Gly Gly Ser Arg Thr
                805
```

<210> 27
<211> 694
<212> PRT
<213> Homo sapiens

<400> 27

```
Met Gly Ala Pro Ala Cys Ala Leu Ala Leu Cys Val Ala Val Ala Ile
 1               5                  10                  15
Val Ala Gly Ala Ser Ser Glu Ser Leu Gly Thr Glu Gln Arg Val Val
            20                  25                  30
Gly Arg Ala Ala Glu Val Pro Gly Pro Glu Pro Gly Gln Gln Glu Gln
        35                  40                  45
Leu Val Phe Gly Ser Gly Asp Ala Val Glu Leu Ser Cys Pro Pro Pro
    50                  55                  60
Gly Gly Gly Pro Met Gly Pro Thr Val Trp Val Lys Asp Gly Thr Gly
65                  70                  75                  80
Leu Val Pro Ser Glu Arg Val Leu Val Gly Pro Gln Arg Leu Gln Val
                85                  90                  95
Leu Asn Ala Ser His Glu Asp Ser Gly Ala Tyr Ser Cys Arg Gln Arg
            100                 105                 110
Leu Thr Gln Arg Val Leu Cys His Phe Ser Val Arg Val Thr Asp Ala
        115                 120                 125
Pro Ser Ser Gly Asp Asp Glu Asp Gly Glu Asp Glu Ala Glu Asp Thr
    130                 135                 140
Gly Val Asp Thr Gly Ala Pro Tyr Trp Thr Arg Pro Glu Arg Met Asp
145                 150                 155                 160
Lys Lys Leu Leu Ala Val Pro Ala Ala Asn Thr Val Arg Phe Arg Cys
                165                 170                 175
Pro Ala Ala Gly Asn Pro Thr Pro Ser Ile Ser Trp Leu Lys Asn Gly
            180                 185                 190
Arg Glu Phe Arg Gly Glu His Arg Ile Gly Gly Ile Lys Leu Arg His
        195                 200                 205
Gln Gln Trp Ser Leu Val Met Glu Ser Val Val Pro Ser Asp Arg Gly
    210                 215                 220
Asn Tyr Thr Cys Val Val Glu Asn Lys Phe Gly Ser Ile Arg Gln Thr
225                 230                 235                 240
Tyr Thr Leu Asp Val Leu Glu Arg Ser Pro His Arg Pro Ile Leu Gln
                245                 250                 255
Ala Gly Leu Pro Ala Asn Gln Thr Ala Val Leu Gly Ser Asp Val Glu
```

```
            260                 265                 270
Phe His Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile Gln Trp Leu
        275                 280                 285
Lys His Val Glu Val Asn Gly Ser Lys Val Gly Pro Asp Gly Thr Pro
    290                 295                 300
Tyr Val Thr Val Leu Lys Val Ser Leu Glu Ser Asn Ala Ser Met Ser
305                 310                 315                 320
Ser Asn Thr Pro Leu Val Arg Ile Ala Arg Leu Ser Ser Gly Glu Gly
                325                 330                 335
Pro Thr Leu Ala Asn Val Ser Glu Leu Glu Leu Pro Ala Asp Pro Lys
            340                 345                 350
Trp Glu Leu Ser Arg Ala Arg Leu Thr Leu Gly Lys Pro Leu Gly Glu
        355                 360                 365
Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Ile Gly Ile Asp Lys
    370                 375                 380
Asp Arg Ala Ala Lys Pro Val Thr Val Ala Val Lys Met Leu Lys Asp
385                 390                 395                 400
Asp Ala Thr Asp Lys Asp Leu Ser Asp Leu Val Ser Glu Met Glu Met
                405                 410                 415
Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn Leu Leu Gly Ala
            420                 425                 430
Cys Thr Gln Gly Gly Pro Leu Tyr Val Leu Val Glu Tyr Ala Ala Lys
        435                 440                 445
Gly Asn Leu Arg Glu Phe Leu Arg Ala Arg Arg Pro Pro Gly Leu Asp
    450                 455                 460
Tyr Ser Phe Asp Thr Cys Lys Pro Pro Glu Glu Gln Leu Thr Phe Lys
465                 470                 475                 480
Asp Leu Val Ser Cys Ala Tyr Gln Val Ala Arg Gly Met Glu Tyr Leu
                485                 490                 495
Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala Arg Asn Val Leu
            500                 505                 510
Val Thr Glu Asp Asn Val Met Lys Ile Ala Asp Phe Gly Leu Ala Arg
        515                 520                 525
Asp Val His Asn Leu Asp Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu
    530                 535                 540
Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp Arg Val Tyr Thr
545                 550                 555                 560
His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu Trp Glu Ile Phe
                565                 570                 575
Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val Glu Glu Leu Phe
            580                 585                 590
Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ala Asn Cys Thr
        595                 600                 605
His Asp Leu Tyr Met Ile Met Arg Glu Cys Trp His Ala Ala Pro Ser
    610                 615                 620
Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu Asp Arg Val Leu
625                 630                 635                 640
Thr Val Thr Ser Thr Asp Glu Tyr Leu Asp Leu Ser Ala Pro Phe Glu
                645                 650                 655
Gln Tyr Ser Pro Gly Gly Gln Asp Thr Pro Ser Ser Ser Ser Ser Gly
            660                 665                 670
Asp Asp Ser Val Phe Ala His Asp Leu Leu Pro Pro Ala Pro Pro Ser
        675                 680                 685
Ser Gly Gly Ser Arg Thr
    690
```

<210> 28
<211> 604

<212> PRT
<213> Homo sapiens

<400> 28

```
Ala Gln Arg Arg Lys Glu Arg Glu Glu Leu Ala Gln Gln Tyr Glu Ala
 1               5                  10                  15
Ile Leu Arg Glu Cys Gly His Gly Arg Phe Gln Trp Thr Leu Tyr Phe
            20                  25                  30
Val Leu Gly Leu Ala Leu Met Ala Asp Gly Val Glu Val Phe Val Val
        35                  40                  45
Gly Phe Val Leu Pro Ser Ala Glu Lys Asp Met Cys Leu Ser Asp Ser
    50                  55                  60
Asn Lys Gly Met Leu Gly Leu Ile Val Tyr Leu Gly Met Met Val Gly
65                  70                  75                  80
Ala Phe Leu Trp Gly Gly Leu Ala Asp Arg Leu Gly Arg Arg Gln Cys
                85                  90                  95
Leu Leu Ile Ser Leu Ser Val Asn Ser Val Phe Ala Phe Phe Ser Ser
            100                 105                 110
Phe Val Gln Gly Tyr Gly Thr Phe Leu Phe Cys Arg Leu Leu Ser Gly
        115                 120                 125
Val Gly Ile Gly Gly Ser Ile Pro Ile Val Phe Ser Tyr Phe Ser Glu
    130                 135                 140
Phe Leu Ala Gln Glu Lys Arg Gly Glu His Leu Ser Trp Leu Cys Met
145                 150                 155                 160
Phe Trp Met Ile Gly Gly Val Tyr Ala Ala Ala Met Ala Trp Ala Ile
                165                 170                 175
Ile Pro His Tyr Gly Trp Ser Phe Gln Met Gly Ser Ala Tyr Gln Phe
            180                 185                 190
His Ser Trp Arg Val Phe Val Leu Val Cys Ala Phe Pro Ser Val Phe
        195                 200                 205
Ala Ile Gly Ala Leu Thr Thr Gln Pro Glu Ser Pro Arg Phe Phe Leu
    210                 215                 220
Glu Asn Gly Lys His Asp Glu Ala Trp Met Val Leu Lys Gln Val His
225                 230                 235                 240
Asp Thr Asn Met Arg Ala Lys Gly His Pro Glu Arg Val Phe Ser Val
                245                 250                 255
Thr His Ile Lys Thr Ile His Gln Glu Asp Glu Leu Ile Glu Ile Gln
            260                 265                 270
Ser Asp Thr Gly Thr Trp Tyr Gln Arg Trp Gly Val Arg Ala Leu Ser
        275                 280                 285
Leu Gly Gly Gln Val Trp Gly Asn Phe Leu Ser Cys Phe Gly Pro Glu
    290                 295                 300
Tyr Arg Arg Ile Thr Leu Met Met Met Gly Val Trp Phe Thr Met Ser
305                 310                 315                 320
Phe Ser Tyr Tyr Gly Leu Thr Val Trp Phe Pro Asp Met Ile Arg His
                325                 330                 335
Leu Gln Ala Val Asp Tyr Ala Ser Arg Thr Lys Val Phe Pro Gly Glu
            340                 345                 350
Arg Val Glu His Val Thr Phe Asn Phe Thr Leu Glu Asn Gln Ile His
        355                 360                 365
Arg Gly Gly Gln Tyr Phe Asn Asp Lys Phe Ile Gly Leu Arg Leu Lys
    370                 375                 380
Ser Val Ser Phe Glu Asp Ser Leu Phe Glu Glu Cys Tyr Phe Glu Asp
385                 390                 395                 400
Val Thr Ser Ser Asn Thr Phe Phe Arg Asn Cys Thr Phe Ile Asn Thr
                405                 410                 415
Val Phe Tyr Asn Thr Asp Leu Phe Glu Tyr Lys Phe Val Asn Ser Arg
            420                 425                 430
Leu Ile Asn Ser Thr Phe Leu His Asn Lys Glu Gly Cys Pro Leu Asp
```

```
        435                 440                 445
Val Thr Gly Thr Gly Glu Gly Ala Tyr Met Val Tyr Phe Val Ser Phe
    450                 455                 460
Leu Gly Thr Leu Ala Val Leu Pro Gly Asn Ile Val Ser Ala Leu Leu
465                 470                 475                 480
Met Asp Lys Ile Gly Arg Leu Arg Met Leu Ala Gly Ser Ser Val Met
                485                 490                 495
Ser Cys Val Ser Cys Phe Phe Leu Ser Phe Gly Asn Ser Glu Ser Ala
            500                 505                 510
Met Ile Ala Leu Leu Cys Leu Phe Gly Gly Val Ser Ile Ala Ser Trp
        515                 520                 525
Asn Ala Leu Asp Val Leu Thr Val Glu Leu Tyr Pro Ser Asp Lys Arg
    530                 535                 540
Thr Thr Ala Phe Gly Phe Leu Asn Ala Leu Cys Lys Leu Ala Ala Val
545                 550                 555                 560
Leu Gly Ile Ser Ile Phe Thr Ser Phe Val Gly Ile Thr Lys Ala Ala
                565                 570                 575
Pro Ile Leu Phe Ala Ser Ala Ala Leu Ala Leu Gly Ser Ser Leu Ala
            580                 585                 590
Leu Lys Leu Pro Glu Thr Arg Gly Gln Val Leu Gln
        595                 600
```

<210> 29
<211> 683
<212> PRT
<213> Homo sapiens

<400> 29

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Met Asp Asp Tyr Lys Tyr Gln Asp Asn Tyr Gly Gly Tyr Ala Pro Ser
 1                5                  10                  15
Asp Gly Tyr Tyr Arg Gly Asn Glu Ser Asn Pro Glu Glu Asp Ala Gln
            20                  25                  30
Ser Asp Val Thr Glu Gly His Asp Glu Glu Asp Glu Ile Tyr Glu Gly
        35                  40                  45
Glu Tyr Gln Gly Ile Pro His Pro Asp Asp Val Lys Ala Lys Gln Ala
    50                  55                  60
Lys Met Ala Pro Ser Arg Met Asp Ser Leu Arg Gly Gln Thr Asp Leu
65                  70                  75                  80
Met Ala Glu Arg Leu Glu Asp Glu Glu Gln Leu Ala His Gln Tyr Glu
                85                  90                  95
Thr Ile Met Asp Glu Cys Gly His Gly Arg Phe Gln Trp Ile Leu Phe
            100                 105                 110
Phe Val Leu Gly Leu Ala Leu Met Ala Asp Gly Val Glu Val Phe Val
        115                 120                 125
Val Ser Phe Ala Leu Pro Ser Ala Glu Lys Asp Met Cys Leu Ser Ser
    130                 135                 140
Ser Lys Lys Gly Met Leu Gly Met Ile Val Tyr Leu Gly Met Met Ala
145                 150                 155                 160
Gly Ala Phe Ile Leu Gly Gly Leu Ala Asp Lys Leu Gly Arg Lys Arg
                165                 170                 175
Val Leu Ser Met Ser Leu Ala Val Asn Ala Ser Phe Ala Ser Leu Ser
            180                 185                 190
Ser Phe Val Gln Gly Tyr Gly Ala Phe Leu Phe Cys Arg Leu Ile Ser
        195                 200                 205
Gly Ile Gly Ile Gly Gly Ala Leu Pro Ile Val Phe Ala Tyr Phe Ser
    210                 215                 220
Glu Phe Leu Ser Arg Glu Lys Arg Gly Glu His Leu Ser Trp Leu Gly
225                 230                 235                 240
```

```
Ile Phe Trp Met Thr Gly Gly Leu Tyr Ala Ser Ala Met Ala Trp Ser
                245                 250                 255
Ile Ile Pro His Tyr Gly Trp Gly Phe Ser Met Gly Thr Asn Tyr His
            260                 265                 270
Phe His Ser Trp Arg Val Phe Val Ile Val Cys Ala Leu Pro Cys Thr
        275                 280                 285
Val Ser Met Val Ala Leu Lys Phe Met Pro Glu Ser Pro Arg Phe Leu
    290                 295                 300
Leu Glu Met Gly Lys His Asp Glu Ala Trp Met Ile Leu Lys Gln Val
305                 310                 315                 320
His Asp Thr Asn Met Arg Ala Lys Gly Thr Pro Glu Lys Val Phe Thr
                325                 330                 335
Val Ser Asn Ile Lys Thr Pro Lys Gln Met Asp Glu Phe Ile Glu Ile
            340                 345                 350
Gln Ser Ser Thr Gly Thr Trp Tyr Gln Arg Trp Leu Val Arg Phe Lys
        355                 360                 365
Thr Ile Phe Lys Gln Val Trp Asp Asn Ala Leu Tyr Cys Val Met Gly
    370                 375                 380
Pro Tyr Arg Met Asn Thr Leu Ile Leu Ala Val Val Trp Phe Ala Met
385                 390                 395                 400
Ala Phe Ser Tyr Tyr Gly Leu Thr Val Trp Phe Pro Asp Met Ile Arg
                405                 410                 415
Tyr Phe Gln Asp Glu Glu Tyr Lys Ser Lys Met Lys Val Phe Phe Gly
            420                 425                 430
Glu His Val Tyr Gly Ala Thr Ile Asn Phe Thr Met Glu Asn Gln Ile
        435                 440                 445
His Gln His Gly Lys Leu Val Asn Asp Lys Phe Thr Arg Met Tyr Phe
    450                 455                 460
Lys His Val Leu Phe Glu Asp Thr Phe Phe Asp Glu Cys Tyr Phe Glu
465                 470                 475                 480
Asp Val Thr Ser Thr Asp Thr Tyr Phe Lys Asn Cys Thr Ile Glu Ser
                485                 490                 495
Thr Ile Phe Tyr Asn Thr Asp Leu Tyr Glu His Lys Phe Ile Asn Cys
            500                 505                 510
Arg Phe Ile Asn Ser Thr Phe Leu Glu Gln Lys Glu Gly Cys His Met
        515                 520                 525
Asp Leu Glu Gln Asp Asn Asp Phe Leu Ile Tyr Leu Val Ser Phe Leu
    530                 535                 540
Gly Ser Leu Ser Val Leu Pro Gly Asn Ile Ile Ser Ala Leu Leu Met
545                 550                 555                 560
Asp Arg Ile Gly Arg Leu Lys Met Ile Gly Gly Ser Met Leu Ile Ser
                565                 570                 575
Ala Val Cys Cys Phe Phe Leu Phe Phe Gly Asn Ser Glu Ser Ala Met
            580                 585                 590
Ile Gly Trp Gln Cys Leu Phe Cys Gly Thr Ser Ile Ala Ala Trp Asn
        595                 600                 605
Ala Leu Asp Val Ile Thr Val Glu Leu Tyr Pro Thr Asn Gln Arg Ala
    610                 615                 620
Thr Ala Phe Gly Ile Leu Asn Gly Leu Cys Lys Phe Gly Ala Ile Leu
625                 630                 635                 640
Gly Asn Thr Ile Phe Ala Ser Phe Val Gly Ile Thr Lys Val Val Pro
                645                 650                 655
Ile Leu Leu Ala Ala Ala Ser Leu Val Gly Gly Gly Leu Ile Ala Leu
            660                 665                 670
Arg Leu Pro Glu Thr Arg Glu Gln Val Leu Ile
        675                 680
```

<210> 30

<211> 727
<212> PRT
<213> Homo sapiens

<400> 30

Met	Glu	Asp	Ser	Tyr	Lys	Asp	Arg	Thr	Ser	Leu	Met	Lys	Gly	Ala	Lys
1				5				10						15	
Asp	Ile	Ala	Arg	Glu	Val	Lys	Lys	Gln	Thr	Val	Lys	Lys	Val	Asn	Gln
			20					25					30		
Ala	Val	Asp	Arg	Ala	Gln	Asp	Glu	Tyr	Thr	Gln	Arg	Ser	Tyr	Ser	Arg
		35					40					45			
Phe	Gln	Asp	Glu	Glu	Asp	Asp	Asp	Asp	Tyr	Tyr	Pro	Ala	Gly	Glu	Thr
	50					55					60				
Tyr	Asn	Gly	Glu	Ala	Asn	Asp	Asp	Glu	Gly	Ser	Ser	Glu	Ala	Thr	Glu
65					70					75					80
Gly	His	Asp	Glu	Asp	Asp	Glu	Ile	Tyr	Glu	Gly	Glu	Tyr	Gln	Gly	Ile
				85					90					95	
Pro	Ser	Met	Asn	Gln	Ala	Lys	Asp	Ser	Ile	Val	Ser	Val	Gly	Gln	Pro
			100					105					110		
Lys	Gly	Asp	Glu	Tyr	Lys	Asp	Arg	Arg	Glu	Leu	Glu	Ser	Glu	Arg	Arg
		115					120					125			
Ala	Asp	Glu	Glu	Glu	Leu	Ala	Gln	Gln	Tyr	Glu	Leu	Ile	Ile	Gln	Glu
	130					135					140				
Cys	Gly	His	Gly	Arg	Phe	Gln	Trp	Ala	Leu	Phe	Phe	Val	Leu	Gly	Met
145					150					155					160
Ala	Leu	Met	Ala	Asp	Gly	Val	Glu	Val	Phe	Val	Val	Gly	Phe	Val	Leu
				165					170					175	
Pro	Ser	Ala	Glu	Thr	Asp	Leu	Cys	Ile	Pro	Asn	Ser	Gly	Ser	Gly	Trp
			180					185					190		
Leu	Gly	Ser	Ile	Val	Tyr	Leu	Gly	Met	Met	Val	Gly	Ala	Phe	Phe	Trp
		195					200					205			
Gly	Gly	Leu	Ala	Asp	Lys	Val	Gly	Arg	Lys	Gln	Ser	Leu	Leu	Ile	Cys
	210					215					220				
Met	Ser	Val	Asn	Gly	Phe	Phe	Ala	Phe	Leu	Ser	Ser	Phe	Val	Gln	Gly
225					230					235					240
Tyr	Gly	Phe	Phe	Leu	Phe	Cys	Arg	Leu	Leu	Ser	Gly	Phe	Gly	Ile	Gly
				245					250					255	
Gly	Ala	Ile	Pro	Thr	Val	Phe	Ser	Tyr	Phe	Ala	Glu	Val	Leu	Ala	Arg
			260					265					270		
Glu	Lys	Arg	Gly	Glu	His	Leu	Ser	Trp	Leu	Cys	Met	Phe	Trp	Met	Ile
		275					280					285			
Gly	Gly	Ile	Tyr	Ala	Ser	Ala	Met	Ala	Trp	Ala	Ile	Ile	Pro	His	Tyr
	290					295					300				
Gly	Trp	Ser	Phe	Ser	Met	Gly	Ser	Ala	Tyr	Gln	Phe	His	Ser	Trp	Arg
305					310					315					320
Val	Phe	Val	Ile	Val	Cys	Ala	Leu	Pro	Cys	Val	Ser	Ser	Val	Val	Ala
				325					330					335	
Leu	Thr	Phe	Met	Pro	Glu	Ser	Pro	Arg	Phe	Leu	Leu	Glu	Val	Gly	Lys
			340					345					350		
His	Asp	Glu	Ala	Trp	Met	Ile	Leu	Lys	Leu	Ile	His	Asp	Thr	Asn	Met
		355					360					365			
Arg	Ala	Arg	Gly	Gln	Pro	Glu	Lys	Val	Phe	Thr	Val	Asn	Lys	Ile	Lys
	370					375					380				
Thr	Pro	Lys	Gln	Ile	Asp	Glu	Leu	Ile	Glu	Ile	Glu	Ser	Asp	Thr	Gly
385					390					395					400
Thr	Trp	Tyr	Arg	Arg	Cys	Phe	Val	Arg	Ile	Arg	Thr	Glu	Leu	Tyr	Gly
				405					410					415	
Ile	Trp	Leu	Thr	Phe	Met	Arg	Cys	Phe	Asn	Tyr	Pro	Val	Arg	Asp	Asn
			420					425					430		

```
Thr Ile Lys Leu Thr Ile Val Trp Phe Thr Leu Ser Phe Gly Tyr Tyr
        435                 440                 445
Gly Leu Ser Val Trp Phe Pro Asp Val Ile Lys Pro Leu Gln Ser Asp
    450                 455                 460
Glu Tyr Ala Leu Leu Thr Arg Asn Val Glu Arg Asp Lys Tyr Ala Asn
465                 470                 475                 480
Phe Thr Ile Asn Phe Thr Met Glu Asn Gln Ile His Thr Gly Met Glu
                485                 490                 495
Tyr Asp Asn Gly Arg Phe Ile Gly Val Lys Phe Lys Ser Val Thr Phe
            500                 505                 510
Lys Asp Ser Val Phe Lys Ser Cys Thr Phe Glu Asp Val Thr Ser Val
        515                 520                 525
Asn Thr Tyr Phe Lys Asn Cys Thr Phe Ile Asp Thr Val Phe Asp Asn
    530                 535                 540
Thr Asp Phe Glu Pro Tyr Lys Phe Ile Asp Ser Glu Phe Lys Asn Cys
545                 550                 555                 560
Ser Phe Phe His Asn Lys Thr Gly Cys Gln Ile Thr Phe Asp Asp Asp
                565                 570                 575
Tyr Ser Ala Tyr Trp Ile Tyr Phe Val Asn Phe Leu Gly Thr Leu Ala
            580                 585                 590
Val Leu Pro Gly Asn Ile Val Ser Ala Leu Leu Met Asp Arg Ile Gly
        595                 600                 605
Arg Leu Thr Met Leu Gly Gly Ser Met Val Leu Ser Gly Ile Ser Cys
    610                 615                 620
Phe Phe Leu Trp Phe Gly Thr Ser Glu Ser Met Met Ile Gly Met Leu
625                 630                 635                 640
Cys Leu Tyr Asn Gly Leu Thr Ile Ser Ala Trp Asn Ser Leu Asp Val
                645                 650                 655
Val Thr Val Glu Leu Tyr Pro Thr Asp Arg Arg Ala Thr Gly Phe Gly
            660                 665                 670
Phe Leu Asn Ala Leu Cys Lys Ala Ala Ala Val Leu Gly Asn Leu Ile
        675                 680                 685
Phe Gly Ser Leu Val Ser Ile Thr Lys Ser Ile Pro Ile Leu Leu Ala
    690                 695                 700
Ser Thr Val Leu Val Cys Gly Gly Leu Val Gly Leu Cys Leu Pro Asp
705                 710                 715                 720
Thr Arg Thr Gln Val Leu Met
                725
```

<210> 31
<211> 742
<212> PRT
<213> Homo sapiens

<400> 31

```
Met Glu Glu Gly Phe Arg Asp Arg Ala Ala Phe Ile Arg Gly Ala Lys
 1               5                  10                  15
Asp Ile Ala Lys Glu Val Lys Lys His Ala Ala Lys Lys Val Val Lys
            20                  25                  30
Gly Leu Asp Arg Val Gln Asp Glu Tyr Ser Arg Arg Ser Tyr Ser Arg
        35                  40                  45
Phe Glu Glu Glu Asp Asp Asp Asp Asp Phe Pro Ala Pro Ser Asp Gly
    50                  55                  60
Tyr Tyr Arg Gly Glu Gly Thr Gln Asp Glu Glu Glu Gly Gly Ala Ser
65                  70                  75                  80
Ser Asp Ala Thr Glu Gly His Asp Glu Asp Asp Glu Ile Tyr Glu Gly
                85                  90                  95
Glu Tyr Gln Asp Ile Pro Arg Ala Glu Ser Gly Gly Lys Gly Glu Arg
```

```
            100                 105                 110
Met Ala Asp Gly Ala Pro Leu Ala Gly Val Arg Gly Gly Leu Ser Asp
        115                 120                 125
Gly Glu Gly Pro Pro Gly Gly Arg Gly Glu Ala Gln Arg Arg Lys Glu
    130                 135                 140
Arg Glu Glu Leu Ala Gln Gln Tyr Glu Ala Ile Leu Arg Glu Cys Gly
145                 150                 155                 160
His Gly Arg Phe Gln Trp Thr Leu Tyr Phe Val Leu Gly Leu Ala Leu
                165                 170                 175
Met Ala Asp Gly Val Glu Val Phe Val Val Gly Phe Val Leu Pro Ser
            180                 185                 190
Ala Glu Lys Asp Met Cys Leu Ser Asp Ser Asn Lys Gly Met Leu Gly
        195                 200                 205
Leu Ile Val Tyr Leu Gly Met Met Val Gly Ala Phe Leu Trp Gly Gly
    210                 215                 220
Leu Ala Asp Arg Leu Gly Arg Arg Gln Cys Leu Leu Ile Ser Leu Ser
225                 230                 235                 240
Val Asn Ser Val Phe Ala Phe Phe Ser Ser Phe Val Gln Gly Tyr Gly
                245                 250                 255
Thr Phe Leu Phe Cys Arg Leu Leu Ser Gly Val Gly Ile Gly Gly Ser
            260                 265                 270
Ile Pro Ile Val Phe Ser Tyr Phe Ser Glu Phe Leu Ala Gln Glu Lys
        275                 280                 285
Arg Gly Glu His Leu Ser Trp Leu Cys Met Phe Trp Met Ile Gly Gly
    290                 295                 300
Val Tyr Ala Ala Ala Met Ala Trp Ala Ile Ile Pro His Tyr Gly Trp
305                 310                 315                 320
Ser Phe Gln Met Gly Ser Ala Tyr Gln Phe His Ser Trp Arg Val Phe
                325                 330                 335
Val Leu Val Cys Ala Phe Pro Ser Val Phe Ala Ile Gly Ala Leu Thr
            340                 345                 350
Thr Gln Pro Glu Ser Pro Arg Phe Phe Leu Glu Asn Gly Lys His Asp
        355                 360                 365
Glu Ala Trp Met Val Leu Lys Gln Val His Asp Thr Asn Met Arg Ala
    370                 375                 380
Lys Gly His Pro Glu Arg Val Phe Ser Val Thr His Ile Lys Thr Ile
385                 390                 395                 400
His Gln Glu Asp Glu Leu Ile Glu Ile Gln Ser Asp Thr Gly Thr Trp
                405                 410                 415
Tyr Gln Arg Trp Gly Val Arg Ala Leu Ser Leu Gly Gly Gln Val Trp
            420                 425                 430
Gly Asn Phe Leu Ser Cys Phe Gly Pro Glu Tyr Arg Arg Ile Thr Leu
        435                 440                 445
Met Met Met Gly Val Trp Phe Thr Met Ser Phe Ser Tyr Tyr Gly Leu
    450                 455                 460
Thr Val Trp Phe Pro Asp Met Ile Arg His Leu Gln Ala Val Asp Tyr
465                 470                 475                 480
Ala Ser Arg Thr Lys Val Phe Pro Gly Glu Arg Val Gly His Val Thr
                485                 490                 495
Phe Asn Phe Thr Leu Glu Asn Gln Ile His Arg Gly Gly Gln Tyr Phe
            500                 505                 510
Asn Asp Lys Phe Ile Gly Leu Arg Leu Lys Ser Val Ser Phe Glu Asp
        515                 520                 525
Ser Leu Phe Glu Glu Cys Tyr Phe Glu Asp Val Thr Ser Ser Asn Thr
    530                 535                 540
Phe Phe Arg Asn Cys Thr Phe Ile Asn Thr Val Phe Tyr Asn Thr Asp
545                 550                 555                 560
Leu Phe Glu Tyr Lys Phe Val Asn Ser Arg Leu Ile Asn Ser Thr Phe
                565                 570                 575
```

```
Leu His Asn Lys Glu Gly Cys Pro Leu Asp Val Thr Gly Thr Gly Glu
            580                 585                 590
Gly Ala Tyr Met Val Tyr Phe Val Ser Phe Leu Gly Thr Leu Ala Val
        595                 600                 605
Leu Pro Gly Asn Ile Val Ser Ala Leu Leu Met Asp Lys Ile Gly Arg
    610                 615                 620
Leu Arg Met Leu Ala Gly Ser Ser Val Met Ser Cys Val Ser Cys Phe
625                 630                 635                 640
Phe Leu Ser Phe Gly Asn Ser Glu Ser Ala Met Ile Ala Leu Leu Cys
                645                 650                 655
Leu Phe Gly Gly Val Ser Ile Ala Ser Trp Asn Ala Leu Asp Val Leu
            660                 665                 670
Thr Val Glu Leu Tyr Pro Ser Asp Lys Arg Thr Thr Ala Phe Gly Phe
        675                 680                 685
Leu Asn Ala Leu Cys Lys Leu Ala Ala Val Leu Gly Ile Ser Ile Phe
    690                 695                 700
Thr Ser Phe Val Gly Ile Thr Lys Ala Ala Pro Ile Leu Phe Ala Ser
705                 710                 715                 720
Ala Ala Leu Ala Leu Gly Ser Ser Leu Ala Leu Lys Leu Pro Glu Thr
                725                 730                 735
Arg Gly Gln Val Leu Gln
            740
```

<210> 32
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 32
Arg Ile Asp Glu Ala Asn Gln
 1   5

<210> 33
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 33
Thr Arg Ile Asp Glu Ala Asn Gln
 1   5

<210> 34
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 34
Lys Thr Arg Ile Asp Glu Ala Asn Gln
1 5

<210> 35
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 35
Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln
1 5 10

<210> 36
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 36
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln
1 5 10

<210> 37
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 37
Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln
1 5 10

<210> 38
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxylated carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<221> SITE
<222> (13)...(13)
<223> carboxylated glutamine

<400> 38
Cys Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln
1 5 10

<210> 39
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 39
Arg Ile Asp Glu Ala Asn Lys
1 5

<210> 40
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 40
Ala Arg Ile Asp Glu Ala Asn Lys
1 5

<210> 41
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 41
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<210> 42
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

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<212> PRT
<213> Artificial Sequence

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1 5 10

<210> 44
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

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<210> 45
<211> 13
<212> PRT
<213> Artificial Sequence

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<223> SNAP-25 antigen having a free carboxylated carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<221> SITE
<222> (13)...(13)
<223> Carboxylated lysine

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<220>
<223> SNAP-25 antigen

<400> 46
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1 5 10

<210> 47
<211> 11
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<213> Artificial Sequence

<220>
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<211> 88
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35 40 45
Gly Asn Leu Arg His Met Ala Leu Asp Met Gly Asn Glu Ile Asp Thr
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<212> PRT
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<220>
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35 40 45
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50 55 60
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<210> 50
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<212> PRT
<213> Artificial Sequence

<220>
<223> BirA peptide

<400> 50
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<210> 51
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<212> DNA
<213> Artificial Sequence

<220>
<223> pQBI-25/GFP-BoNT/A-LC expression construct.

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<211> 682
<212> PRT
<213> Artificial Sequence

<220>
<223> GFP-BoNT/A light chain amino acid sequence.

<400> 52

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Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
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Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
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His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
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Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
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Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
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Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
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Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
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Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
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Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
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Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
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Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ile Asp
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Pro Val Asn Gly Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Ala Gly
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Gln Met Gln Pro Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val
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Ile Pro Glu Arg Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn
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Pro Pro Pro Glu Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr
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Thr Glu Leu Lys Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro
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Ser Ala Asp Ile Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val
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His Asp Ala Lys Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu
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<212> DNA
<213> Artificial Sequence

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<223> pQBI-25/GFP expression construct.

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<210> 54
<211> 245
<212> PRT
<213> Artificial Sequence

<220>
<223> GFP amino acid sequence.

<400> 54

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            20                  25                  30
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
    50                  55                  60
Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
65                  70                  75                  80
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
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    130                 135                 140
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160
Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ile Asp
225                 230                 235                 240
Gly Gly Gly Gly Gly
                245
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<210> 55
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> G-spacer flexible spacer

<400> 55
Gly Gly Gly Gly
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<210> 56
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> G-spacer flexible spacer

<400> 56
Gly Gly Gly Gly Ser
 1   5

<210> 57
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> A-spacer flexible spacer

<400> 57
Ala Ala Ala Ala
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<210> 58
<211> 5
<212> PRT

<213> Artificial Sequence

<220>
<223> A-spacer flexible spacer

<400> 58
Ala Ala Ala Ala Val
1 5

<210> 59
<211> 821
<212> PRT
<213> Homo sapiens

<400> 59
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20 25 30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
35 40 45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
50 55 60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65 70 75 80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
85 90 95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
100 105 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
115 120 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
130 135 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145 150 155 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
165 170 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
180 185 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
195 200 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
210 215 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225 230 235 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
245 250 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
260 265 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
275 280 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
290 295 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys Ala Ala Gly Val Asn Thr Thr
305 310 315 320
Asp Lys Glu Ile Glu Val Leu Tyr Ile Arg Asn Val Thr Phe Glu Asp
325 330 335
Ala Gly Glu Tyr Thr Cys Leu Ala Gly Asn Ser Ile Gly Ile Ser Phe

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            340                 345                 350
His Ser Ala Trp Leu Thr Val Leu Pro Ala Pro Gly Arg Glu Lys Glu
        355                 360                 365
Ile Thr Ala Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile Gly
    370                 375                 380
Val Phe Leu Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg Met
385                 390                 395                 400
Lys Asn Thr Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val His
                405                 410                 415
Lys Leu Thr Lys Arg Ile Pro Leu Arg Arg Gln Val Thr Val Ser Ala
            420                 425                 430
Glu Ser Ser Ser Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile Thr
        435                 440                 445
Thr Arg Leu Ser Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val Ser
    450                 455                 460
Glu Tyr Glu Leu Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp Lys
465                 470                 475                 480
Leu Thr Leu Gly Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val Val
                485                 490                 495
Met Ala Glu Ala Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala Val
            500                 505                 510
Thr Val Ala Val Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp Leu
        515                 520                 525
Ser Asp Leu Val Ser Glu Met Glu Met Met Lys Met Ile Gly Lys His
    530                 535                 540
Lys Asn Ile Ile Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro Leu
545                 550                 555                 560
Tyr Val Ile Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu
                565                 570                 575
Arg Ala Arg Arg Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn Arg
            580                 585                 590
Val Pro Glu Glu Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr Tyr
        595                 600                 605
Gln Leu Ala Arg Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile His
    610                 615                 620
Arg Asp Leu Ala Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val Met
625                 630                 635                 640
Lys Ile Ala Asp Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp Tyr
                645                 650                 655
Tyr Lys Lys Thr Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala Pro
            660                 665                 670
Glu Ala Leu Phe Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp Ser
        675                 680                 685
Phe Gly Val Leu Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro Tyr
    690                 695                 700
Pro Gly Ile Pro Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly His
705                 710                 715                 720
Arg Met Asp Lys Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met Met
                725                 730                 735
Arg Asp Cys Trp His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln
            740                 745                 750
Leu Val Glu Asp Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu Glu
        755                 760                 765
Tyr Leu Asp Leu Ser Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr Pro
    770                 775                 780
Asp Thr Arg Ser Ser Cys Ser Ser Gly Asp Asp Ser Val Phe Ser Pro
785                 790                 795                 800
Asp Pro Met Pro Tyr Glu Pro Cys Leu Pro Gln Tyr Pro His Ile Asn
                805                 810                 815
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Gly Ser Val Lys Thr
820

<210> 60
<211> 822
<212> PRT
<213> Homo sapiens

<400> 60
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
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20 25 30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
35 40 45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
50 55 60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65 70 75 80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
85 90 95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
100 105 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
115 120 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
130 135 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145 150 155 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
165 170 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
180 185 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
195 200 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
210 215 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225 230 235 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
245 250 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
260 265 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
275 280 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
290 295 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys His Ser Gly Ile Asn Ser Ser
305 310 315 320
Asn Ala Glu Val Leu Ala Leu Phe Asn Val Thr Glu Ala Asp Ala Gly
325 330 335
Glu Tyr Ile Cys Lys Val Ser Asn Tyr Ile Gly Gln Ala Asn Gln Ser
340 345 350
Ala Trp Leu Thr Val Leu Pro Lys Gln Gln Ala Pro Gly Arg Glu Lys
355 360 365
Glu Ile Thr Ala Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile
370 375 380
Gly Val Phe Leu Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg

385 390 395 400
Met Lys Asn Thr Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val
405 410 415
His Lys Leu Thr Lys Arg Ile Pro Leu Arg Arg Gln Val Thr Val Ser
420 425 430
Ala Glu Ser Ser Ser Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile
435 440 445
Thr Thr Arg Leu Ser Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val
450 455 460
Ser Glu Tyr Glu Leu Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp
465 470 475 480
Lys Leu Thr Leu Gly Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val
485 490 495
Val Met Ala Glu Ala Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala
500 505 510
Val Thr Val Ala Val Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp
515 520 525
Leu Ser Asp Leu Val Ser Glu Met Glu Met Met Lys Met Ile Gly Lys
530 535 540
His Lys Asn Ile Ile Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro
545 550 555 560
Leu Tyr Val Ile Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr
565 570 575
Leu Arg Ala Arg Arg Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn
580 585 590
Arg Val Pro Glu Glu Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr
595 600 605
Tyr Gln Leu Ala Arg Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile
610 615 620
His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val
625 630 635 640
Met Lys Ile Ala Asp Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp
645 650 655
Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala
660 665 670
Pro Glu Ala Leu Phe Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp
675 680 685
Ser Phe Gly Val Leu Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro
690 695 700
Tyr Pro Gly Ile Pro Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly
705 710 715 720
His Arg Met Asp Lys Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met
725 730 735
Met Arg Asp Cys Trp His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys
740 745 750
Gln Leu Val Glu Asp Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu
755 760 765
Glu Tyr Leu Asp Leu Ser Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr
770 775 780
Pro Asp Thr Arg Ser Ser Cys Ser Ser Gly Asp Asp Ser Val Phe Ser
785 790 795 800
Pro Asp Pro Met Pro Tyr Glu Pro Cys Leu Pro Gln Tyr Pro His Ile
805 810 815
Asn Gly Ser Val Lys Thr
820

<210> 61
<211> 769

<212> PRT
<213> Homo sapiens

<400> 61

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            20                  25                  30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
        35                  40                  45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
    50                  55                  60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65                  70                  75                  80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
                85                  90                  95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
            100                 105                 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
        115                 120                 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
    130                 135                 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145                 150                 155                 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
                165                 170                 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
            180                 185                 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
        195                 200                 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
    210                 215                 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225                 230                 235                 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
                245                 250                 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
            260                 265                 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
        275                 280                 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
    290                 295                 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys His Ser Gly Ile Asn Ser Ser
305                 310                 315                 320
Asn Ala Glu Val Leu Ala Leu Phe Asn Val Thr Glu Ala Asp Ala Gly
                325                 330                 335
Glu Tyr Ile Cys Lys Val Ser Asn Tyr Ile Gly Gln Ala Asn Gln Ser
            340                 345                 350
Ala Trp Leu Thr Val Leu Pro Lys Gln Gln Ala Pro Gly Arg Glu Lys
        355                 360                 365
Glu Ile Thr Ala Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile
    370                 375                 380
Gly Val Phe Leu Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg
385                 390                 395                 400
Met Lys Asn Thr Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val
                405                 410                 415
His Lys Leu Thr Lys Arg Ile Pro Leu Arg Arg Gln Val Thr Val Ser
            420                 425                 430
Ala Glu Ser Ser Ser Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile
```

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        435                 440                 445
Thr Thr Arg Leu Ser Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val
    450                 455                 460
Ser Glu Tyr Glu Leu Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp
465                 470                 475                 480
Lys Leu Thr Leu Gly Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val
                485                 490                 495
Val Met Ala Glu Ala Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala
            500                 505                 510
Val Thr Val Ala Val Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp
        515                 520                 525
Leu Ser Asp Leu Val Ser Glu Met Glu Met Met Lys Met Ile Gly Lys
    530                 535                 540
His Lys Asn Ile Ile Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro
545                 550                 555                 560
Leu Tyr Val Ile Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr
                565                 570                 575
Leu Arg Ala Arg Arg Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn
            580                 585                 590
Arg Val Pro Glu Glu Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr
        595                 600                 605
Tyr Gln Leu Ala Arg Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile
    610                 615                 620
His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val
625                 630                 635                 640
Met Lys Ile Ala Asp Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp
                645                 650                 655
Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala
            660                 665                 670
Pro Glu Ala Leu Phe Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp
        675                 680                 685
Ser Phe Gly Val Leu Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro
    690                 695                 700
Tyr Pro Gly Ile Pro Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly
705                 710                 715                 720
His Arg Met Asp Lys Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met
                725                 730                 735
Met Arg Asp Cys Trp His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys
            740                 745                 750
Gln Leu Val Glu Asp Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu
        755                 760                 765
Ile
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<210> 62
<211> 709
<212> PRT
<213> Homo sapiens

<400> 62

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Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1               5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
        35                  40                  45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
    50                  55                  60
```

```
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65                  70                  75                  80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
                85                  90                  95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
            100                 105                 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
        115                 120                 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
    130                 135                 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145                 150                 155                 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
                165                 170                 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
            180                 185                 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
        195                 200                 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
    210                 215                 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225                 230                 235                 240
Asn His Thr Tyr His Leu Asp Val Val Ala Pro Gly Arg Glu Lys Glu
                245                 250                 255
Ile Thr Ala Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile Gly
            260                 265                 270
Val Phe Leu Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg Met
        275                 280                 285
Lys Asn Thr Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val His
    290                 295                 300
Lys Leu Thr Lys Arg Ile Pro Leu Arg Arg Gln Val Thr Val Ser Ala
305                 310                 315                 320
Glu Ser Ser Ser Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile Thr
                325                 330                 335
Thr Arg Leu Ser Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val Ser
            340                 345                 350
Glu Tyr Glu Leu Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp Lys
        355                 360                 365
Leu Thr Leu Gly Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val Val
    370                 375                 380
Met Ala Glu Ala Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala Val
385                 390                 395                 400
Thr Val Ala Val Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp Leu
                405                 410                 415
Ser Asp Leu Val Ser Glu Met Glu Met Met Lys Met Ile Gly Lys His
            420                 425                 430
Lys Asn Ile Ile Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro Leu
        435                 440                 445
Tyr Val Ile Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu
    450                 455                 460
Arg Ala Arg Arg Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn Arg
465                 470                 475                 480
Val Pro Glu Glu Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr Tyr
                485                 490                 495
Gln Leu Ala Arg Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile His
            500                 505                 510
Arg Asp Leu Ala Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val Met
        515                 520                 525
Lys Ile Ala Asp Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp Tyr
```

```
    530                 535                 540
Tyr Lys Lys Thr Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala Pro
545                 550                 555                 560
Glu Ala Leu Phe Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp Ser
                565                 570                 575
Phe Gly Val Leu Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro Tyr
            580                 585                 590
Pro Gly Ile Pro Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly His
        595                 600                 605
Arg Met Asp Lys Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met Met
    610                 615                 620
Arg Asp Cys Trp His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln
625                 630                 635                 640
Leu Val Glu Asp Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu Glu
                645                 650                 655
Tyr Leu Asp Leu Ser Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr Pro
            660                 665                 670
Asp Thr Arg Ser Ser Cys Ser Ser Gly Asp Asp Ser Val Phe Ser Pro
        675                 680                 685
Asp Pro Met Pro Tyr Glu Pro Cys Leu Pro Gln Tyr Pro His Ile Asn
    690                 695                 700
Gly Ser Val Lys Thr
705
```

<210> 63
<211> 707
<212> PRT
<213> Homo sapiens

<400> 63

```
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1               5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Asp Ala Ile Ser Ser Gly Asp Asp Glu Asp Asp Thr
        35                  40                  45
Asp Gly Ala Glu Asp Phe Val Ser Glu Asn Ser Asn Asn Lys Arg Ala
    50                  55                  60
Pro Tyr Trp Thr Asn Thr Glu Lys Met Glu Lys Arg Leu His Ala Val
65                  70                  75                  80
Pro Ala Ala Asn Thr Val Lys Phe Arg Cys Pro Ala Gly Gly Asn Pro
                85                  90                  95
Met Pro Thr Met Arg Trp Leu Lys Asn Gly Lys Glu Phe Lys Gln Glu
            100                 105                 110
His Arg Ile Gly Gly Tyr Lys Val Arg Asn Gln His Trp Ser Leu Ile
        115                 120                 125
Met Glu Ser Val Val Pro Ser Asp Lys Gly Asn Tyr Thr Cys Val Val
    130                 135                 140
Glu Asn Glu Tyr Gly Ser Ile Asn His Thr Tyr His Leu Asp Val Val
145                 150                 155                 160
Glu Arg Ser Pro His Arg Pro Ile Leu Gln Ala Gly Leu Pro Ala Asn
                165                 170                 175
Ala Ser Thr Val Val Gly Gly Asp Val Glu Phe Val Cys Lys Val Tyr
            180                 185                 190
Ser Asp Ala Gln Pro His Ile Gln Trp Ile Lys His Val Glu Lys Asn
        195                 200                 205
Gly Ser Lys Tyr Gly Pro Asp Gly Leu Pro Tyr Leu Lys Val Leu Lys
    210                 215                 220
```

```
Ala Ala Gly Val Asn Thr Thr Asp Lys Glu Ile Glu Val Leu Tyr Ile
225                 230                 235                 240
Arg Asn Val Thr Phe Glu Asp Ala Gly Glu Tyr Thr Cys Leu Ala Gly
                245                 250                 255
Asn Ser Ile Gly Ile Ser Phe His Ser Ala Trp Leu Thr Val Leu Pro
            260                 265                 270
Ala Pro Gly Arg Glu Lys Glu Ile Thr Ala Ser Pro Asp Tyr Leu Glu
        275                 280                 285
Ile Ala Ile Tyr Cys Ile Gly Val Phe Leu Ile Ala Cys Met Val Val
    290                 295                 300
Thr Val Ile Leu Cys Arg Met Lys Asn Thr Thr Lys Lys Pro Asp Phe
305                 310                 315                 320
Ser Ser Gln Pro Ala Val His Lys Leu Thr Lys Arg Ile Pro Leu Arg
                325                 330                 335
Arg Gln Val Thr Val Ser Ala Glu Ser Ser Ser Ser Met Asn Ser Asn
            340                 345                 350
Thr Pro Leu Val Arg Ile Thr Thr Arg Leu Ser Ser Thr Ala Asp Thr
        355                 360                 365
Pro Met Leu Ala Gly Val Ser Glu Tyr Glu Leu Pro Glu Asp Pro Lys
    370                 375                 380
Trp Glu Phe Pro Arg Asp Lys Leu Thr Leu Gly Lys Pro Leu Gly Glu
385                 390                 395                 400
Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Val Gly Ile Asp Lys
                405                 410                 415
Asp Lys Pro Lys Glu Ala Val Thr Val Ala Val Lys Met Leu Lys Asp
            420                 425                 430
Asp Ala Thr Glu Lys Asp Leu Ser Asp Leu Val Ser Glu Met Glu Met
        435                 440                 445
Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn Leu Leu Gly Ala
    450                 455                 460
Cys Thr Gln Asp Gly Pro Leu Tyr Val Ile Val Glu Tyr Ala Ser Lys
465                 470                 475                 480
Gly Asn Leu Arg Glu Tyr Leu Arg Ala Arg Arg Pro Pro Gly Met Glu
                485                 490                 495
Tyr Ser Tyr Asp Ile Asn Arg Val Pro Glu Glu Gln Met Thr Phe Lys
            500                 505                 510
Asp Leu Val Ser Cys Thr Tyr Gln Leu Ala Arg Gly Met Glu Tyr Leu
        515                 520                 525
Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala Arg Asn Val Leu
    530                 535                 540
Val Thr Glu Asn Asn Val Met Lys Ile Ala Asp Phe Gly Leu Ala Arg
545                 550                 555                 560
Asp Ile Asn Asn Ile Asp Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu
                565                 570                 575
Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp Arg Val Tyr Thr
            580                 585                 590
His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Met Trp Glu Ile Phe
        595                 600                 605
Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val Glu Glu Leu Phe
    610                 615                 620
Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ala Asn Cys Thr
625                 630                 635                 640
Asn Glu Leu Tyr Met Met Met Arg Asp Cys Trp His Ala Val Pro Ser
                645                 650                 655
Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu Asp Arg Ile Leu
            660                 665                 670
Thr Leu Thr Thr Asn Glu Glu Glu Lys Lys Val Ser Gly Ala Val Asp
        675                 680                 685
Cys His Lys Pro Pro Cys Asn Pro Ser His Leu Pro Cys Val Leu Ala
```

690 695 700
Val Asp Gln
705

<210> 64
<211> 706
<212> PRT
<213> Homo sapiens

<400> 64
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
1 5 10 15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
20 25 30
Leu Glu Pro Glu Gly Ala Pro Tyr Trp Thr Asn Thr Glu Lys Met Glu
35 40 45
Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys Phe Arg Cys
50 55 60
Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu Lys Asn Gly
65 70 75 80
Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys Val Arg Asn
85 90 95
Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser Asp Lys Gly
100 105 110
Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile Asn His Thr
115 120 125
Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro Ile Leu Gln
130 135 140
Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly Asp Val Glu
145 150 155 160
Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile Gln Trp Ile
165 170 175
Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp Gly Leu Pro
180 185 190
Tyr Leu Lys Val Leu Lys Ala Ala Gly Val Asn Thr Thr Asp Lys Glu
195 200 205
Ile Glu Val Leu Tyr Ile Arg Asn Val Thr Phe Glu Asp Ala Gly Glu
210 215 220
Tyr Thr Cys Leu Ala Gly Asn Ser Ile Gly Ile Ser Phe His Ser Ala
225 230 235 240
Trp Leu Thr Val Leu Pro Ala Pro Gly Arg Glu Lys Glu Ile Thr Ala
245 250 255
Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile Gly Val Phe Leu
260 265 270
Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg Met Lys Asn Thr
275 280 285
Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val His Lys Leu Thr
290 295 300
Lys Arg Ile Pro Leu Arg Arg Gln Val Thr Val Ser Ala Glu Ser Ser
305 310 315 320
Ser Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile Thr Thr Arg Leu
325 330 335
Ser Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val Ser Glu Tyr Glu
340 345 350
Leu Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp Lys Leu Thr Leu
355 360 365
Gly Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val Val Met Ala Glu
370 375 380

```
Ala Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala Val Thr Val Ala
385                 390                 395                 400
Val Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp Leu Ser Asp Leu
                405                 410                 415
Val Ser Glu Met Glu Met Met Lys Met Ile Gly Lys His Lys Asn Ile
            420                 425                 430
Ile Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro Leu Tyr Val Ile
        435                 440                 445
Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu Arg Ala Arg
    450                 455                 460
Arg Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn Arg Val Pro Glu
465                 470                 475                 480
Glu Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr Tyr Gln Leu Ala
                485                 490                 495
Arg Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile His Arg Asp Leu
            500                 505                 510
Ala Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val Met Lys Ile Ala
        515                 520                 525
Asp Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp Tyr Tyr Lys Lys
    530                 535                 540
Thr Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala Pro Glu Ala Leu
545                 550                 555                 560
Phe Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp Ser Phe Gly Val
                565                 570                 575
Leu Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile
            580                 585                 590
Pro Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp
        595                 600                 605
Lys Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met Met Arg Asp Cys
    610                 615                 620
Trp His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu
625                 630                 635                 640
Asp Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu Glu Tyr Leu Asp
                645                 650                 655
Leu Ser Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr Pro Asp Thr Arg
            660                 665                 670
Ser Ser Cys Ser Ser Gly Asp Asp Ser Val Phe Ser Pro Asp Pro Met
        675                 680                 685
Pro Tyr Glu Pro Cys Leu Pro Gln Tyr Pro His Ile Asn Gly Ser Val
    690                 695                 700
Lys Thr
705
```

<210> 65
<211> 705
<212> PRT
<213> Homo sapiens

<400> 65

```
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1               5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
        35                  40                  45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
    50                  55                  60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
```

65 70 75 80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
85 90 95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
100 105 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
115 120 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
130 135 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145 150 155 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
165 170 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
180 185 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
195 200 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
210 215 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225 230 235 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
245 250 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
260 265 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
275 280 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
290 295 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys Val Ser Ala Glu Ser Ser Ser
305 310 315 320
Ser Met Asn Ser Asn Thr Pro Leu Val Arg Ile Thr Thr Arg Leu Ser
325 330 335
Ser Thr Ala Asp Thr Pro Met Leu Ala Gly Val Ser Glu Tyr Glu Leu
340 345 350
Pro Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp Lys Leu Thr Leu Gly
355 360 365
Lys Pro Leu Gly Glu Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala
370 375 380
Val Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala Val Thr Val Ala Val
385 390 395 400
Lys Met Leu Lys Asp Asp Ala Thr Glu Lys Asp Leu Ser Asp Leu Val
405 410 415
Ser Glu Met Glu Met Met Lys Met Ile Gly Lys His Lys Asn Ile Ile
420 425 430
Asn Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro Leu Tyr Val Ile Val
435 440 445
Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu Arg Ala Arg Arg
450 455 460
Pro Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn Arg Val Pro Glu Glu
465 470 475 480
Gln Met Thr Phe Lys Asp Leu Val Ser Cys Thr Tyr Gln Leu Ala Arg
485 490 495
Gly Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala
500 505 510
Ala Arg Asn Val Leu Val Thr Glu Asn Asn Val Met Lys Ile Ala Asp
515 520 525
Phe Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp Tyr Tyr Lys Lys Thr
530 535 540

```
Thr Asn Gly Arg Leu Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe
545                 550                 555                 560
Asp Arg Val Tyr Thr His Gln Ser Asp Val Trp Ser Phe Gly Val Leu
                565                 570                 575
Met Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro
            580                 585                 590
Val Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys
        595                 600                 605
Pro Ala Asn Cys Thr Asn Glu Leu Tyr Met Met Met Arg Asp Cys Trp
    610                 615                 620
His Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp
625                 630                 635                 640
Leu Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu Glu Tyr Leu Asp Leu
                645                 650                 655
Ser Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr Pro Asp Thr Arg Ser
            660                 665                 670
Ser Cys Ser Ser Gly Asp Asp Ser Val Phe Ser Pro Asp Pro Met Pro
        675                 680                 685
Tyr Glu Pro Cys Leu Pro Gln Tyr Pro His Ile Asn Gly Ser Val Lys
    690                 695                 700
Thr
705
```

<210> 66
<211> 704
<212> PRT
<213> Homo sapiens

<400> 66

```
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1               5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Gly Ala Pro Tyr Trp Thr Asn Thr Glu Lys Met Glu
        35                  40                  45
Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys Phe Arg Cys
    50                  55                  60
Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu Lys Asn Gly
65                  70                  75                  80
Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys Val Arg Asn
                85                  90                  95
Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser Asp Lys Gly
            100                 105                 110
Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile Asn His Thr
        115                 120                 125
Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro Ile Leu Gln
    130                 135                 140
Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly Asp Val Glu
145                 150                 155                 160
Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile Gln Trp Ile
                165                 170                 175
Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp Gly Leu Pro
            180                 185                 190
Tyr Leu Lys Val Leu Lys Ala Ala Gly Val Asn Thr Thr Asp Lys Glu
        195                 200                 205
Ile Glu Val Leu Tyr Ile Arg Asn Val Thr Phe Glu Asp Ala Gly Glu
    210                 215                 220
Tyr Thr Cys Leu Ala Gly Asn Ser Ile Gly Ile Ser Phe His Ser Ala
```

```
225                 230                 235                 240
Trp Leu Thr Val Leu Pro Ala Pro Gly Arg Glu Lys Glu Ile Thr Ala
                245                 250                 255
Ser Pro Asp Tyr Leu Glu Ile Ala Ile Tyr Cys Ile Gly Val Phe Leu
            260                 265                 270
Ile Ala Cys Met Val Val Thr Val Ile Leu Cys Arg Met Lys Asn Thr
        275                 280                 285
Thr Lys Lys Pro Asp Phe Ser Ser Gln Pro Ala Val His Lys Leu Thr
    290                 295                 300
Lys Arg Ile Pro Leu Arg Arg Gln Val Ser Ala Glu Ser Ser Ser Ser
305                 310                 315                 320
Met Asn Ser Asn Thr Pro Leu Val Arg Ile Thr Thr Arg Leu Ser Ser
                325                 330                 335
Thr Ala Asp Thr Pro Met Leu Ala Gly Val Ser Glu Tyr Glu Leu Pro
            340                 345                 350
Glu Asp Pro Lys Trp Glu Phe Pro Arg Asp Lys Leu Thr Leu Gly Lys
        355                 360                 365
Pro Leu Gly Glu Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Val
    370                 375                 380
Gly Ile Asp Lys Asp Lys Pro Lys Glu Ala Val Thr Val Ala Val Lys
385                 390                 395                 400
Met Leu Lys Asp Asp Ala Thr Glu Lys Asp Leu Ser Asp Leu Val Ser
                405                 410                 415
Glu Met Glu Met Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn
            420                 425                 430
Leu Leu Gly Ala Cys Thr Gln Asp Gly Pro Leu Tyr Val Ile Val Glu
        435                 440                 445
Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu Arg Ala Arg Arg Pro
    450                 455                 460
Pro Gly Met Glu Tyr Ser Tyr Asp Ile Asn Arg Val Pro Glu Glu Gln
465                 470                 475                 480
Met Thr Phe Lys Asp Leu Val Ser Cys Thr Tyr Gln Leu Ala Arg Gly
                485                 490                 495
Met Glu Tyr Leu Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala
            500                 505                 510
Arg Asn Val Leu Val Thr Glu Asn Asn Val Met Lys Ile Ala Asp Phe
        515                 520                 525
Gly Leu Ala Arg Asp Ile Asn Asn Ile Asp Tyr Tyr Lys Lys Thr Thr
    530                 535                 540
Asn Gly Arg Leu Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp
545                 550                 555                 560
Arg Val Tyr Thr His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Met
                565                 570                 575
Trp Glu Ile Phe Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val
            580                 585                 590
Glu Glu Leu Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro
        595                 600                 605
Ala Asn Cys Thr Asn Glu Leu Tyr Met Met Met Arg Asp Cys Trp His
    610                 615                 620
Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu
625                 630                 635                 640
Asp Arg Ile Leu Thr Leu Thr Thr Asn Glu Glu Tyr Leu Asp Leu Ser
                645                 650                 655
Gln Pro Leu Glu Gln Tyr Ser Pro Ser Tyr Pro Asp Thr Arg Ser Ser
            660                 665                 670
Cys Ser Ser Gly Asp Asp Ser Val Phe Ser Pro Asp Pro Met Pro Tyr
        675                 680                 685
Glu Pro Cys Leu Pro Gln Tyr Pro His Ile Asn Gly Ser Val Lys Thr
    690                 695                 700
```

<210> 67
<211> 680
<212> PRT
<213> Homo sapiens

<400> 67
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
1 5 10 15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
20 25 30
Leu Glu Pro Glu Asp Ala Ile Ser Ser Gly Asp Asp Glu Asp Asp Thr
35 40 45
Asp Gly Ala Glu Asp Phe Val Ser Glu Asn Ser Asn Asn Lys Arg Ala
50 55 60
Pro Tyr Trp Thr Asn Thr Glu Lys Met Glu Lys Arg Leu His Ala Val
65 70 75 80
Pro Ala Ala Asn Thr Val Lys Phe Arg Cys Pro Ala Gly Gly Asn Pro
85 90 95
Met Pro Thr Met Arg Trp Leu Lys Asn Gly Lys Glu Phe Lys Gln Glu
100 105 110
His Arg Ile Gly Gly Tyr Lys Val Arg Asn Gln His Trp Ser Leu Ile
115 120 125
Met Glu Ser Val Val Pro Ser Asp Lys Gly Asn Tyr Thr Cys Val Val
130 135 140
Glu Asn Glu Tyr Gly Ser Ile Asn His Thr Tyr His Leu Asp Val Val
145 150 155 160
Glu Arg Ser Pro His Arg Pro Ile Leu Gln Ala Gly Leu Pro Ala Asn
165 170 175
Ala Ser Thr Val Val Gly Gly Asp Val Glu Phe Val Cys Lys Val Tyr
180 185 190
Ser Asp Ala Gln Pro His Ile Gln Trp Ile Lys His Val Glu Lys Asn
195 200 205
Gly Ser Lys Tyr Gly Pro Asp Gly Leu Pro Tyr Leu Lys Val Leu Lys
210 215 220
His Ser Gly Ile Asn Ser Ser Asn Ala Glu Val Leu Ala Leu Phe Asn
225 230 235 240
Val Thr Glu Ala Asp Ala Gly Glu Tyr Ile Cys Lys Val Ser Asn Tyr
245 250 255
Ile Gly Gln Ala Asn Gln Ser Ala Trp Leu Thr Val Leu Pro Lys Gln
260 265 270
Gln Ala Pro Gly Arg Glu Lys Glu Ile Thr Ala Ser Pro Asp Tyr Leu
275 280 285
Glu Ile Ala Ile Tyr Cys Ile Gly Val Phe Leu Ile Ala Cys Met Val
290 295 300
Val Thr Val Ile Leu Cys Arg Met Lys Asn Thr Thr Lys Lys Pro Asp
305 310 315 320
Phe Ser Ser Gln Pro Ala Val His Lys Leu Thr Lys Arg Ile Pro Leu
325 330 335
Arg Arg Gln Val Thr Val Ser Ala Glu Ser Ser Ser Ser Met Asn Ser
340 345 350
Asn Thr Pro Leu Val Arg Ile Thr Thr Arg Leu Ser Ser Thr Ala Asp
355 360 365
Thr Pro Met Leu Ala Gly Val Ser Glu Tyr Glu Leu Pro Glu Asp Pro
370 375 380
Lys Trp Glu Phe Pro Arg Asp Lys Leu Thr Leu Gly Lys Pro Leu Gly
385 390 395 400
Glu Gly Cys Phe Gly Gln Val Val Met Ala Glu Ala Val Gly Ile Asp

405 410 415
Lys Asp Lys Pro Lys Glu Ala Val Thr Val Ala Val Lys Met Leu Lys
420 425 430
Asp Asp Ala Thr Glu Lys Asp Leu Ser Asp Leu Val Ser Glu Met Glu
435 440 445
Met Met Lys Met Ile Gly Lys His Lys Asn Ile Ile Asn Leu Leu Gly
450 455 460
Ala Cys Thr Gln Asp Gly Pro Leu Tyr Val Ile Val Glu Tyr Ala Ser
465 470 475 480
Lys Gly Asn Leu Arg Glu Tyr Leu Arg Ala Arg Arg Pro Pro Gly Met
485 490 495
Glu Tyr Ser Tyr Asp Ile Asn Arg Val Pro Glu Glu Gln Met Thr Phe
500 505 510
Lys Asp Leu Val Ser Cys Thr Tyr Gln Leu Ala Arg Gly Met Glu Tyr
515 520 525
Leu Ala Ser Gln Lys Cys Ile His Arg Asp Leu Ala Ala Arg Asn Val
530 535 540
Leu Val Thr Glu Asn Asn Val Met Lys Ile Ala Asp Phe Gly Leu Ala
545 550 555 560
Arg Asp Ile Asn Asn Ile Asp Tyr Tyr Lys Lys Thr Thr Asn Gly Arg
565 570 575
Leu Pro Val Lys Trp Met Ala Pro Glu Ala Leu Phe Asp Arg Val Tyr
580 585 590
Thr His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Met Trp Glu Ile
595 600 605
Phe Thr Leu Gly Gly Ser Pro Tyr Pro Gly Ile Pro Val Glu Glu Leu
610 615 620
Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ala Asn Cys
625 630 635 640
Thr Asn Glu Leu Tyr Met Met Met Arg Asp Cys Trp His Ala Val Pro
645 650 655
Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp Leu Asp Arg Ile
660 665 670
Leu Thr Leu Thr Thr Asn Glu Ile
675 680

<210> 68
<211> 396
<212> PRT
<213> Homo sapiens

<400> 68
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
1 5 10 15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
20 25 30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
35 40 45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
50 55 60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65 70 75 80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
85 90 95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
100 105 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
115 120 125

```
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
    130                 135                 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145                 150                 155                 160
Lys Thr Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
                165                 170                 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
            180                 185                 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
        195                 200                 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
    210                 215                 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225                 230                 235                 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
                245                 250                 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
            260                 265                 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
        275                 280                 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
    290                 295                 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys Ala Ala Gly Val Asn Thr Thr
305                 310                 315                 320
Asp Lys Glu Ile Glu Val Leu Tyr Ile Arg Asn Val Thr Phe Glu Asp
                325                 330                 335
Ala Gly Glu Tyr Thr Cys Leu Ala Gly Asn Ser Ile Gly Ile Ser Phe
            340                 345                 350
His Ser Ala Trp Leu Thr Val Leu Pro Gly Ile Tyr Cys Ser Phe Ser
        355                 360                 365
Leu Gly Phe Phe Pro Phe Ser Trp Leu Thr Ala Ile Lys Leu Thr Gln
    370                 375                 380
Leu Leu Leu Ser Glu Met Ala Pro Phe Ile Leu Ala
385                 390                 395


<210> 69
<211> 317
<212> PRT
<213> Homo sapiens

<400> 69
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1                  5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
        35                  40                  45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
    50                  55                  60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65                  70                  75                  80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
                85                  90                  95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
            100                 105                 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
        115                 120                 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
```

```
    130                 135                 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145                 150                 155                 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
                165                 170                 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
            180                 185                 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
        195                 200                 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
    210                 215                 220
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225                 230                 235                 240
Asn His Thr Tyr His Leu Asp Val Val Glu Arg Ser Pro His Arg Pro
                245                 250                 255
Ile Leu Gln Ala Gly Leu Pro Ala Asn Ala Ser Thr Val Val Gly Gly
            260                 265                 270
Asp Val Glu Phe Val Cys Lys Val Tyr Ser Asp Ala Gln Pro His Ile
        275                 280                 285
Gln Trp Ile Lys His Val Glu Lys Asn Gly Ser Lys Tyr Gly Pro Asp
    290                 295                 300
Gly Leu Pro Tyr Leu Lys Val Leu Lys Val Arg Thr Phe
305                 310                 315


<210> 70
<211> 266
<212> PRT
<213> Homo sapiens

<400> 70
Met Val Ser Trp Gly Arg Phe Ile Cys Leu Val Val Val Thr Met Ala
 1               5                  10                  15
Thr Leu Ser Leu Ala Arg Pro Ser Phe Ser Leu Val Glu Asp Thr Thr
            20                  25                  30
Leu Glu Pro Glu Glu Pro Pro Thr Lys Tyr Gln Ile Ser Gln Pro Glu
        35                  40                  45
Val Tyr Val Ala Ala Pro Gly Glu Ser Leu Glu Val Arg Cys Leu Leu
    50                  55                  60
Lys Asp Ala Ala Val Ile Ser Trp Thr Lys Asp Gly Val His Leu Gly
65                  70                  75                  80
Pro Asn Asn Arg Thr Val Leu Ile Gly Glu Tyr Leu Gln Ile Lys Gly
                85                  90                  95
Ala Thr Pro Arg Asp Ser Gly Leu Tyr Ala Cys Thr Ala Ser Arg Thr
            100                 105                 110
Val Asp Ser Glu Thr Trp Tyr Phe Met Val Asn Val Thr Asp Ala Ile
        115                 120                 125
Ser Ser Gly Asp Asp Glu Asp Asp Thr Asp Gly Ala Glu Asp Phe Val
    130                 135                 140
Ser Glu Asn Ser Asn Asn Lys Arg Ala Pro Tyr Trp Thr Asn Thr Glu
145                 150                 155                 160
Lys Met Glu Lys Arg Leu His Ala Val Pro Ala Ala Asn Thr Val Lys
                165                 170                 175
Phe Arg Cys Pro Ala Gly Gly Asn Pro Met Pro Thr Met Arg Trp Leu
            180                 185                 190
Lys Asn Gly Lys Glu Phe Lys Gln Glu His Arg Ile Gly Gly Tyr Lys
        195                 200                 205
Val Arg Asn Gln His Trp Ser Leu Ile Met Glu Ser Val Val Pro Ser
    210                 215                 220
```

```
Asp Lys Gly Asn Tyr Thr Cys Val Val Glu Asn Glu Tyr Gly Ser Ile
225                 230                 235                 240
Asn His Thr Tyr His Leu Asp Val Val Gly Glu Ser Ala Ser Pro Arg
                245                 250                 255
Val Ala Ala Ala Tyr Gln Pro Ile Leu Ala
            260                 265


<210> 71
<211> 336
<212> DNA
<213> Mus musculus

<400> 71
caggtgaagc tgcaggagtc tggcgctgag ttggtgaaac ctggggcttc agtgaagata 60
tcctgcaagg cttctggcta catcttcact gaccatgctc ttcactgggt gaggcagaag 120
cctgaacagg gcctggaatg gattgggtat atttttcccg gaaatggtaa tattgagtac 180
aatgagaagt tcaagggcaa ggccacactg actgcagaca aatcctccag tactgcctac 240
atgcagctca acagcctgac atctggagat tctgcaatgt atttctgtaa aaagatggac 300
tactggggcc aagggaccac ggtcaccgtc tcctca                           336

<210> 72
<211> 111
<212> PRT
<213> Mus musculus

<400> 72
Val Lys Leu Gln Glu Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser
 1               5                  10                  15
Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ile Phe Thr Asp His Ala
            20                  25                  30
Leu His Trp Val Arg Gln Lys Pro Glu Gln Gly Leu Glu Trp Ile Gly
        35                  40                  45
Tyr Ile Phe Pro Gly Asn Gly Asn Ile Glu Tyr Asn Glu Lys Phe Lys
    50                  55                  60
Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met
65                  70                  75                  80
Gln Leu Asn Ser Leu Thr Ser Gly Asp Ser Ala Met Tyr Phe Cys Lys
                85                  90                  95
Lys Met Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
            100                 105                 110


<210> 73
<211> 336
<212> DNA
<213> Mus musculus

<400> 73
caggtgaagc tgcaggagtc tggcgctgag ttggtgaaac ctggggcttc agtgaagatc 60
tcctgcaagg cttctggtta caccttcact gaccattcta ttcactgggt gaagcagaag 120
cctggacagg gcctagaatg gattggatat ctttttcccg gaaatggtaa ttttgaatat 180
aatgagaaat tcaagggcaa ggccacactg actgcagaca aatcctccag cactgcctac 240
atgcacctca acagcctgac atctgaggat tctgcagtgt atttctgtaa aaagatggac 300
tactggggcc aagggaccac ggtcaccgtc tcctca                           336

<210> 74
<211> 111
<212> PRT
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<213> Mus musculus

<400> 74
Val Lys Leu Gln Glu Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser
1 5 10 15
Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ser
20 25 30
Ile His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile Gly
35 40 45
Tyr Leu Phe Pro Gly Asn Gly Asn Phe Glu Tyr Asn Glu Lys Phe Lys
50 55 60
Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met
65 70 75 80
His Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Lys
85 90 95
Lys Met Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
100 105 110

<210> 75
<211> 336
<212> DNA
<213> Mus musculus

<400> 75
caggttcagc tgcagcagtc cgacgctgag ttggtgaaac ctggggcttc agtgaagata 60
tcctgcaggg cttctggcta caccttcact gaccattcta ttcactgggt gaagcagcag 120
cctggccagg gcctggaatg gatcggatat atttttcccg gaaatggaaa tattgaatac 180
aatgacaaat tcaagggcaa ggccacactg actgcagaca aatcctccgg cactgcctac 240
atgcagctca acagcctgac atctgaggat tctgcagtgt atttctgtaa aaggatgggg 300
tactggggtc aaggaacctc agtcaccgtc tcctca 336

<210> 76
<211> 111
<212> PRT
<213> Mus musculus

<400> 76
Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser
1 5 10 15
Val Lys Ile Ser Cys Arg Ala Ser Gly Tyr Thr Phe Thr Asp His Ser
20 25 30
Ile His Trp Val Lys Gln Gln Pro Gly Gln Gly Leu Glu Trp Ile Gly
35 40 45
Tyr Ile Phe Pro Gly Asn Gly Asn Ile Glu Tyr Asn Asp Lys Phe Lys
50 55 60
Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Gly Thr Ala Tyr Met
65 70 75 80
Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Lys
85 90 95
Arg Met Gly Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
100 105 110

<210> 77
<211> 360
<212> DNA
<213> Mus musculus

<400> 77
caggtcaagc tgcaggagtc tggacctgaa ctggtaaagc ctggggcttc agtgaagatg 60
tcctgcaagg cttctggata cacattcact aactatgtta tacactgggt gaagcaaaag 120
cctgggcagg gccttgagtg gattggatat attaatcctt acaatgatgg ctctaagtac 180
aatgagaagt tcaaaggcaa ggcctcactg acttcagaca aatcctccag cacagcctac 240
atggagctca gcagcctgac ctctgaggac tctgcggtct attactgtgc aagacatctc 300
gctaatacct actactactt tgactactgg ggccaaggga ccacggtcac cgtctcctca 360

<210> 78
<211> 119
<212> PRT
<213> Mus musculus

<400> 78
Val Lys Leu Gln Glu Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser
 1   5   10   15
Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Val
            20   25   30
Ile His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile Gly
        35   40   45
Tyr Ile Asn Pro Tyr Asn Asp Gly Ser Lys Tyr Asn Glu Lys Phe Lys
    50   55   60
Gly Lys Ala Ser Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr Met
65   70   75   80
Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala
                85   90   95
Arg His Leu Ala Asn Thr Tyr Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
            100   105   110
Thr Thr Val Thr Val Ser Ser
        115

<210> 79
<211> 336
<212> DNA
<213> Mus musculus

<400> 79
caggtcaagc tgcaggagtc tggcgctgag ttggtgaaac ctggggcttc agtgaagatc 60
tcctgcaagg cttctggcta caccttcact gaccattcta ttcactgggt gaagcagaag 120
cctggacagg gcctagaatg gattggatat ctttttcccg gaaatggtaa ttttgagtac 180
aatgaaaaat tcaagggcaa ggccacactg actgcagaca aatcctccag cactgtctac 240
atgtacctca acagcctgac atctgaggat tctgcagtgt atttctgtaa aaggatgggg 300
tactggggcc aagggaccac ggtcaccgtc tcctca 336

<210> 80
<211> 111
<212> PRT
<213> Mus musculus

<400> 80
Val Lys Leu Gln Glu Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser
 1   5   10   15
Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ser
            20   25   30
Ile His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile Gly
        35   40   45
Tyr Leu Phe Pro Gly Asn Gly Asn Phe Glu Tyr Asn Glu Lys Phe Lys

```
    50                  55                  60
Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr Met
65                  70                  75                  80
Tyr Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Lys
                85                  90                  95
Arg Met Gly Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
            100                 105                 110
```

<210> 81
<211> 357
<212> DNA
<213> Mus musculus

<400> 81
gtgaagctgc aggagtctgg acctgaactg gtaaagcctg gggcttcagt gaagatgtcc 60
tgcaaggctt ctggatacac attcactaac tatgttatac actgggtgaa gcaaaagcct 120
gggcagggcc ttgagtggat tggatatatt aatccttaca atgatggctc taagtacaat 180
gagaagttca aaggcaaggc ctcactgact tcagacaaat cctccagcac agcctacatg 240
gagctcagca gcctgacctc tgaggactct gcggtctatt actgtgcaag acatctcgct 300
aatacctact actactttga ctactggggc caaggcacca ctctcacagt ctcctca 357

<210> 82
<211> 120
<212> PRT
<213> Mus musculus

<400> 82
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Gln Val Gln Leu Gln Glu Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 1               5                  10                  15
Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
            20                  25                  30
Val Ile His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45
Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Ser Lys Tyr Asn Glu Lys Phe
    50                  55                  60
Lys Gly Lys Ala Ser Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg His Leu Ala Asn Thr Tyr Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110
Gly Thr Thr Leu Thr Val Ser Ser
        115                 120
```

<210> 83
<211> 342
<212> DNA
<213> Mus musculus

<400> 83
gatgttttga tgacccaaac tccactctcc ctgcctgtca gtcttggaga tcaagcctcc 60
atctcttgca gatctagtca gagcattgta catagtaatg gaaacaccta tttagaatgg 120
tacctgcaga aaccaggcca gtctccaaag ctcctgatct acaaagtttc caaccgattt 180
tctggggtcc cagacaggtt cagtggcagt ggatcaggga cagatttcac actcaagatc 240
agcagagtgg aggctgagga tctgggagtt tattactgct ttcaaggttc acatgttcct 300
cctacgttcg gtgctgggac caagctggag ctgaaacggg ct 342

<210> 84
<211> 113
<212> PRT
<213> Mus musculus

<400> 84
Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
1 5 10 15
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Ile Val His Ser
20 25 30
Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45
Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95
Ser His Val Pro Pro Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys
100 105 110
Arg

<210> 85
<211> 324
<212> DNA
<213> Mus musculus

<400> 85
gacatccaga tgactcagtc tccagcctcc ctatctgcat ctgtgggaga aactgtcacc 60
atcacatgtc gaacaactga aaatatttac agttattttg tatggtctca gcagagacag 120
ggaaaatctc ctcagctccg ggtctataat gcaaaatcct tagcagaagg tgtgccatca 180
agtttcaatg tcagtgtatc aggcacacag ttttctctga agatcaatag cctgcagcct 240
gaagattttg ggacttatca ctgtcaacac cattatggta ctccgtacac gttcggaggg 300
gggaccaggc tggaaataag acgg 324

<210> 86
<211> 108
<212> PRT
<213> Mus musculus

<400> 86
Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Glu Thr Val Thr Ile Thr Cys Arg Thr Thr Glu Asn Ile Tyr Ser Tyr
20 25 30
Phe Val Trp Ser Gln Gln Arg Gln Gly Lys Ser Pro Gln Leu Arg Val
35 40 45
Tyr Asn Ala Lys Ser Leu Ala Glu Gly Val Pro Ser Ser Phe Asn Val
50 55 60
Ser Val Ser Gly Thr Gln Phe Ser Leu Lys Ile Asn Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Gly Thr Tyr His Cys Gln His His Tyr Gly Thr Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Arg Leu Glu Ile Arg Arg
100 105

<210> 87
<211> 336
<212> DNA
<213> Mus musculus

<400> 87
gacattgtgc tgacacagtc tcctgcttcc ttagctgtat ctctggggca gagggccacc 60
atctcgtaca gggccagcaa aagtgtcagt acatctggct atagttatat gcactggaac 120
caacagaaac caggacagcc acccagactc ctcatctatc ttgtatccaa cctagaatct 180
ggggtccctg ccaggttcag tggcagtggg tctgggacag acttcaccct caacatccat 240
cctgtggagg aggaggatgc tgcaacctat tactgtcagc acattaggga gcttacacgt 300
tcggaggggg gcaccaagct ggaaatcaaa cggaga 336

<210> 88
<211> 112
<212> PRT
<213> Mus musculus

<400> 88
Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly
 1   5   10   15
Gln Arg Ala Thr Ile Ser Tyr Arg Ala Ser Lys Ser Val Ser Thr Ser
            20   25   30
Gly Tyr Ser Tyr Met His Trp Asn Gln Gln Lys Pro Gly Gln Pro Pro
        35   40   45
Arg Leu Leu Ile Tyr Leu Val Ser Asn Leu Glu Ser Gly Val Pro Ala
    50   55   60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His
65   70   75   80
Pro Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr Cys Gln His Ile Arg
                85   90   95
Glu Leu Thr Arg Ser Glu Gly Gly Thr Lys Leu Glu Ile Lys Arg Arg
            100   105   110

<210> 89
<211> 327
<212> DNA
<213> Mus musculus

<400> 89
gacatcaaga tgacccagtc tccatcctcc atgtatgcat cgctgggaga gagagtcact 60
atcacttgca aggcgagtca ggacattaaa agctatttaa gctggtacca gcagaaacca 120
tggaaatctc ctaagaccct gatctattat gcaacaagct tggcagatgg ggtcccatca 180
agattcagtg gcagtggatc tgggcaagat tattctctaa ccatcagcag cctggagtct 240
gacgatacag caacttatta ctgtctacag catggtgaga gcccgtacac gttcggaggg 300
gggaccaagc tggaaataaa acgggct 327

<210> 90
<211> 108
<212> PRT
<213> Mus musculus

<400> 90
Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Ala Ser Leu Gly
 1   5   10   15
Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Tyr
            20   25   30
Leu Ser Trp Tyr Gln Gln Lys Pro Trp Lys Ser Pro Lys Thr Leu Ile

```
        35                  40                  45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Glu Ser
65                  70                  75                  80
Asp Asp Thr Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Phe
                85                  90                  95
Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
```

<210> 91
<211> 327
<212> DNA
<213> Mus musculus

<400> 91
gatgttgtgc taactcagtc tcctgccacc ctgtctgtga ctccaggaga tagagtcagt 60
ctttcctgca gggccagcca aaatattggc aactacctac actggtatca acagaaatca 120
catgagtctc caaggcttct catcaagtat gcttcccagt ccatctctgg gatcccctcc 180
aggttcagtg gcagtggatc agtcacagat ttcactctca atatcaacag tgtggagact 240
gaagattttg gaatgtattt ctgtcaacag agtgacacct ggcctctcac gttcggtgct 300
gggaccaagc tggagctgaa acgggct 327

<210> 92
<211> 108
<212> PRT
<213> Mus musculus

<400> 92
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Asp Val Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Thr Pro Gly
 1               5                  10                  15
Asp Arg Val Ser Leu Ser Cys Arg Ala Ser Gln Asn Ile Gly Asn Tyr
            20                  25                  30
Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile
        35                  40                  45
Lys Tyr Ala Ser Gln Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Val Thr Asp Phe Thr Leu Asn Ile Asn Ser Val Glu Thr
65                  70                  75                  80
Glu Asp Phe Gly Met Tyr Phe Cys Gln Gln Ser Asp Thr Trp Pro Leu
                85                  90                  95
Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
            100                 105
```

<210> 93
<211> 8
<212> PRT
<213> Mus musculus

<400> 93
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Thr Phe Thr Asp His Ser Ile His
 1               5
```

<210> 94
<211> 8
<212> PRT

<213> Mus musculus

<400> 94
Thr Phe Thr Asn Tyr Val Ile His
1 5

<210> 95
<211> 8
<212> PRT
<213> Mus musculus

<400> 95
Ile Phe Thr Asp His Ala Leu His
1 5

<210> 96
<211> 17
<212> PRT
<213> Mus musculus

<400> 96
Tyr Ile Phe Pro Gly Asn Gly Asn Ile Glu Tyr Asn Asp Lys Phe Lys
1 5 10 15
Gly

<210> 97
<211> 17
<212> PRT
<213> Mus musculus

<400> 97
Tyr Leu Phe Pro Gly Asn Gly Asn Phe Glu Tyr Asn Glu Lys Phe Lys
1 5 10 15
Gly

<210> 98
<211> 17
<212> PRT
<213> Mus musculus

<400> 98
Tyr Ile Asn Pro Tyr Asn Asp Gly Ser Lys Tyr Asn Glu Lys Phe Lys
1 5 10 15
Gly

<210> 99
<211> 17
<212> PRT
<213> Mus musculus

<400> 99

Tyr Ile Phe Pro Gly Asn Gly Asn Ile Glu Tyr Asn Glu Lys Phe Lys
1 5 10 15
Gly

<210> 100
<211> 5
<212> PRT
<213> Mus musculus

<400> 100
Lys Arg Met Gly Tyr
1 5

<210> 101
<211> 5
<212> PRT
<213> Mus musculus

<400> 101
Lys Lys Met Asp Tyr
1 5

<210> 102
<211> 13
<212> PRT
<213> Mus musculus

<400> 102
Ala Arg His Leu Ala Asn Thr Tyr Tyr Tyr Phe Asp Tyr
1 5 10

<210> 103
<211> 16
<212> PRT
<213> Mus musculus

<400> 103
Arg Ser Ser Gln Ser Ile Val His Ser Asn Gly Asn Thr Tyr Leu Glu
1 5 10 15

<210> 104
<211> 11
<212> PRT
<213> Mus musculus

<400> 104
Arg Thr Thr Glu Asn Ile Tyr Ser Tyr Phe Val
1 5 10

<210> 105
<211> 15
<212> PRT

<213> Mus musculus

<400> 105
Arg Ala Ser Lys Ser Val Ser Thr Ser Gly Tyr Ser Tyr Met His
1 5 10 15

<210> 106
<211> 11
<212> PRT
<213> Mus musculus

<400> 106
Lys Ala Ser Gln Asp Ile Lys Ser Tyr Leu Ser
1 5 10

<210> 107
<211> 11
<212> PRT
<213> Mus musculus

<400> 107
Arg Ala Ser Gln Asn Ile Gly Asn Tyr Leu His
1 5 10

<210> 108
<211> 7
<212> PRT
<213> Mus musculus

<400> 108
Lys Val Ser Asn Arg Phe Ser
1 5

<210> 109
<211> 7
<212> PRT
<213> Mus musculus

<400> 109
Asn Ala Lys Ser Leu Ala Glu
1 5

<210> 110
<211> 7
<212> PRT
<213> Mus musculus

<400> 110
Leu Val Ser Asn Leu Glu Ser
1 5

<210> 111
<211> 7

<212> PRT
<213> Mus musculus

<400> 111
Tyr Ala Thr Ser Leu Ala Asp
1 5

<210> 112
<211> 7
<212> PRT
<213> Mus musculus

<400> 112
Tyr Ala Ser Gln Ser Ile Ser
1 5

<210> 113
<211> 9
<212> PRT
<213> Mus musculus

<400> 113
Phe Gln Gly Ser His Val Pro Pro Thr
1 5

<210> 114
<211> 9
<212> PRT
<213> Mus musculus

<400> 114
Gln His His Tyr Gly Thr Pro Tyr Thr
1 5

<210> 115
<211> 9
<212> PRT
<213> Mus musculus

<400> 115
Gln His Ile Arg Glu Leu Thr Arg Ser
1 5

<210> 116
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<211> 2362
<212> DNA
<213> Homo sapiens

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<211> 2053
<212> DNA
<213> Homo sapiens

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<213> Homo sapiens

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<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at
the P1 residue of the scissile bond of the BoNT/A
cleavage site

<400> 147
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1 5

<210> 148
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> SNAP-25 antigen having a free carboxyl-terminus at the P1 residue of the scissile bond of the BoNT/A cleavage site

<400> 148
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