

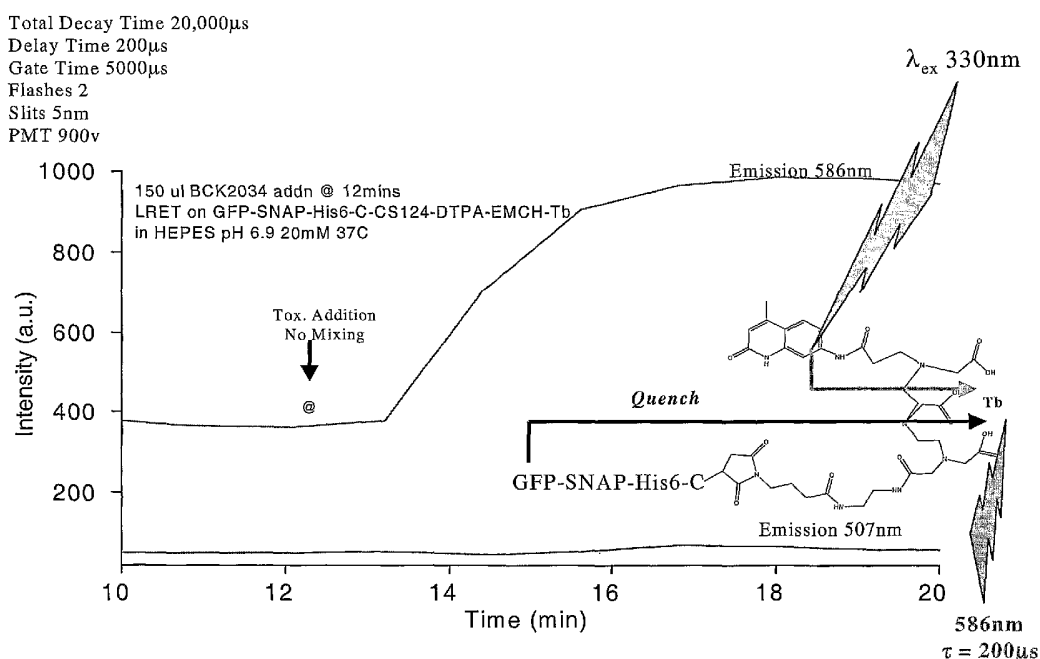


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(54) Title: LANTHANIDE-BASED SUBSTRATES AND METHODS FOR DETERMINING CLOSTRIDIAL TOXIN ACTIVITY



(57) Abstract: The present invention provides a clostridial toxin substrate that contains (a) a lanthanide donor complex; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (c) a clostridial toxin recognition sequence containing a cleavage site that intervenes between the lanthanide donor complex and the acceptor, where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Lanthanide-Based Substrates and Methods for Determining Clostridial Toxin Activity

[01] The present invention relates generally to protease assays, and more specifically, to methods for determining the presence or activity of clostridial toxins such as botulinum toxins and tetanus toxins using substrates containing lanthanides.

[02] The neuromuscular syndrome of tetanus and the rare but potentially fatal disease, botulism, are caused by neurotoxins produced by bacteria of the genus *Clostridium*. These clostridial neurotoxins are highly potent and specific poisons of neural cells, with the human lethal dose of the botulinum toxins on the order of nanograms. Thus, the presence of even minute levels of botulinum toxins in foodstuffs represents a public health hazard that must be avoided through rigorous testing.

[03] However, in spite of their potentially deleterious effects, low controlled doses of botulinum neurotoxins have been successfully used as therapeutics and for some cosmetic applications. In particular, botulinum toxins have been used in the therapeutic management of a variety of focal and segmental dystonias, strabismus, and other conditions in which a reversible depression of cholinergic nerve terminal activity is desired. Established therapeutic uses of botulinum neurotoxins in humans include, without limitation, treatment of blepharospasm, hemifacial spasm, laryngeal dysphonia, focal hyperhidrosis, hypersalivation, oromandibular dystonia, cervical dystonia, torticollis, strabismus, limb dystonia, occupational cramps and myokymia (Rossetto et al., *Toxicon* 39:27-41 (2001)). As an example, intramuscular injection of spastic tissue with small quantities of botulinum neurotoxin A has been used effectively to treat spasticity due to brain injury, spinal cord injury, stroke, multiple sclerosis and cerebral palsy. Additional possible clinical uses of clostridial neurotoxins are currently being investigated.

[04] Given the potential danger associated with small quantities of botulinum toxins in foodstuffs and the need to prepare accurate pharmaceutical formulations, assays for botulinum neurotoxins presently are employed in the food and pharmaceutical industries. The food industry requires assays for the botulinum neurotoxins to validate new food packaging methods and to ensure food safety. The growing clinical use of the botulinum toxins necessitates accurate assays for botulinum neurotoxin activity for product formulation as well as quality control. In both industries, a mouse lethality test currently is the only acceptable assay for botulinum neurotoxin potency.

[05] Unfortunately, the mouse lethality assay suffers from several drawbacks: cost due to the large numbers of laboratory animals required; lack of specificity; potential for inaccuracy unless large animal groups are used; and sacrifice of animal life. Thus, there is a need for new methods based on convenient synthetic substrates that can complement and reduce the need for the mouse lethality assay. The present invention satisfies this need by providing novel assays for determining the presence or activity of a clostridial toxin and provides related advantages as well.

[06] The present invention provides a clostridial toxin substrate that contains (a) a lanthanide donor complex; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (c) a clostridial toxin recognition sequence containing a cleavage site that intervenes between the lanthanide donor complex and the acceptor, where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor.

[07] The present invention further provides a method of determining the presence or activity of a clostridial toxin by (a) treating with a sample, under conditions suitable for clostridial toxin protease activity, a clostridial toxin substrate containing (i) a lanthanide donor complex; (ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (iii) a clostridial toxin recognition sequence containing a cleavage site that intervenes between the lanthanide donor complex and the acceptor, where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor; (b) exciting an antenna of the lanthanide donor complex; and (c) determining resonance energy transfer of the treated substrate relative to a control substrate, where a difference in resonance energy transfer of the treated substrate as compared to the control substrate is indicative of the presence or activity of the clostridial toxin.

[08] Also provided herein is a nucleic acid molecule which contains a nucleotide sequence encoding a clostridial toxin substrate which includes (a), together with a lanthanide ion, a lanthanide donor complex; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (c) a clostridial toxin recognition sequence containing a cleavage site, where the cleavage site intervenes between the lanthanide donor complex and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor.

BRIEF DESCRIPTION OF THE DRAWINGS

[09] Figure 1 shows a schematic of the four steps required for tetanus and botulinum toxin activity in central and peripheral neurons.

[010] Figure 2 shows the subcellular localization and sites of cleavage of SNAP-25, VAMP and syntaxin. VAMP is bound to synaptic vesicle membrane, whereas SNAP-25 and syntaxin are bound to the target plasma membrane. BoNT/A and /E cleave SNAP-25 close to the carboxy-terminus, releasing nine or 26 residues, respectively. BoNT/B, /D, /F, /G and TeNT act on the conserved central portion of VAMP (dotted) and release the amino-terminal portion of VAMP into the cytosol. BoNT/C1 cleaves SNAP-25 close to the carboxy-terminus as well as cleaving syntaxin at a single site near the cytosolic membrane surface. The action of BoNT/B, /C1, /D, /F, /G and TeNT results in release of a large portion

of the cytosolic domain of VAMP or syntaxin, while only a small portion of SNAP-25 is released by selective proteolysis of BoNT/A, /C1 or /E.

[011] Figure 3 shows an alignment of various SNAP-25 proteins. Human SNAP-25 (SEQ ID NO: 1; GenBank accession g4507099; see, also, related human SNAP-25 sequence g2135800); mouse SNAP-25 (SEQ ID NO: 2; GenBank accession G6755588); Drosophila SNAP-25 (SEQ ID NO: 3; GenBank accession g548941); goldfish SNAP-25 (SEQ ID NO: 4; GenBank accession g2133923); sea urchin SNAP-25 (SEQ ID NO: 5; GenBank accession g2707818) and chicken SNAP-25 (SEQ ID NO: 6; GenBank accession g481202) are depicted.

[012] Figure 4 shows an alignment of various VAMP proteins. Human VAMP-1 (SEQ ID NO: 7; GenBank accession g135093); human VAMP-2 (SEQ ID NO: 8; GenBank accession g135094); mouse VAMP-2 (SEQ ID NO: 9; GenBank accession g2501081); bovine VAMP (SEQ ID NO: 10; GenBank accession g89782); frog VAMP (SEQ ID NO: 11; GenBank accession g6094391); and sea urchin VAMP (SEQ ID NO: 12; GenBank accession g5031415) are depicted.

[013] Figure 5 shows an alignment of various syntaxin proteins. Human syntaxin 1A (SEQ ID NO: 13; GenBank accession g15079184), human syntaxin 1B2 (SEQ ID NO: 14; GenBank accession g15072437), mouse syntaxin 1A (SEQ ID NO: 15; GenBank accession g15011853), Drosophila syntaxin 1A (SEQ ID NO: 16; GenBank accession g2501095); C. elegans syntaxin A (SEQ ID NO: 17; GenBank accession g7511662) and sea urchin syntaxin (SEQ ID NO: 18; GenBank accession g13310402) are depicted.

[014] Figure 6 shows a canonical EF-hand containing an α -helix (E, residues 1-11), a lanthanide-binding loop, and a second α -helix (F, residues 19-29). The α -carbons, indicated by *n* (residues 2, 5, 6, 9, 17, 22, 25, 26, and (29)) usually have hydrophobic side chains. They point inward and interact with the homologous residues of a second EF-hand domain, related to the first by a local two-fold axis, to form a hydrophobic core. Ile, Leu, or Val at residue 17 attaches the loop to the hydrophobic core. An asterisk indicates variable residues which are often hydrophilic. Gly at position 15 permits a sharp bend in the lanthanide-binding loop. Residues specifically indicated reflect a strong consensus but are not invariant. The lanthanide ion is coordinated by an oxygen atom, or bridging water molecule, of the side chains of residues 10 (X), 12 (Y), 14 (Z), and 18 (-X). The ligand at vertex -Y is the carbonyl oxygen of residue 16. Typically, residue 21 (-Z) is Glu and is the sixth residue to coordinate the lanthanide ion. See Nakayama and Kretsinger, Annu. Rev. Biophys. Biomol. Struct. 23:473-507 (1994).

[015] Figure 7 shows (A) a schematic of plasmid pQBI GFP-SNAP25₍₁₃₄₋₂₀₆₎-6XHIS-C and (B) the nucleic acid and amino acid sequences (SEQ ID NOS: 19 and 20) of pQBI GFP-SNAP25₍₁₃₄₋₂₀₆₎-6XHIS-C.

[016] Figure 8 shows (A) the absorption spectrum and (B) the excitation (dotted) and emission (bold) spectra of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6C.

[017] Figure 9 shows (A) the UV-VIS absorption spectrum and (B) the emission spectrum using pulse gated excitation at 300 nm of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C-CS124-DTPA-EMCH-Tb.

[018] Figure 10 shows a luminescence resonance energy transfer (LRET) assay of clostridial toxin activity using the lanthanide-based substrate GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C-CS124-DTPA-EMCH-Tb. (A) Quench relief shown by LRET upon addition of dilute reduced bulk BoNT/A at 131 ng/ml cuvette concentration at 37 °C. The terbium emission at 586 nm increased upon addition of toxin. (B) Emission spectrum of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C-CS124-DTPA-EMCH-Tb using pulse gated Xenon excitation at 330 nm before and after turnover. The dotted trace represents gated terbium emission before turnover while the solid trace represents gated terbium emission after turnover.

DETAILED DESCRIPTION

[019] The invention provides novel substrates and methods for determining the presence or activity of clostridial toxins including botulinum toxins of all serotypes as well as tetanus toxins. The novel methods of the invention, which rely on a clostridial toxin substrate containing a lanthanide ion such as terbium, reduce the need for animal toxicity studies and can be used to analyze crude and bulk samples as well as highly purified dichain or single chain toxins or formulated toxin products. The novel lanthanide-based methods of the invention can be performed as homogeneous solution-phase assays and are amenable to automated high-throughput formats. Furthermore, the methods of the invention can be performed as time-resolved assays, which are particularly useful in analyzing samples containing non-specific background fluorescence.

[020] As disclosed herein in Example I, a recombinant fusion protein was prepared containing green fluorescent protein fused to a portion of SNAP-25 and further engineered to contain a carboxy-terminal cysteine. Maleimide chemistry was used to derivatize the carboxy-terminal cysteine of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C with the lumiphore CS124-DTPA-EMCH-Tb. The absorption and emission spectra of the CS124-DTPA-EMCH-Tb labeled GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C are shown in Figures 9A and 9B, respectively. As can be seen in Figure 9B, excitation of the sensitizing group carbostyryl 124 (CS124) at 330 nm resulted in the characteristic long lifetime emission of terbium which yields a series of four prominent sharp bands at 490 nm, 546 nm, 586 nm and 622 nm.

[021] As further disclosed herein in Example II, this clostridial toxin substrate was useful for sensitively assaying for the activity of bulk BoNT/A toxin. In particular, energy transfer between the lanthanide donor complex and GFP was observed by monitoring terbium emission at 586 nm. As shown in Figure 10A, there was a notable increase in luminescence intensity at 586 nm following addition of reduced bulk

BoNT-A toxin, indicative of the relief of quenching between the lanthanide donor complex and GFP. Furthermore, the signal to noise ratio for the emission process was greatly enhanced by utilizing a gated process to monitor the emission as shown in Figure 10B, in which the solid trace represents gated terbium emission before turnover of substrate and the dotted trace represents gated terbium emission after turnover.

[022] In sum, these results indicate that GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C can be derivatized with a commercially available lanthanide donor complex such as CS124-DTPA-EMCH-Tb to produce a clostridial toxin substrate which exhibits quenching between the lanthanide donor complex and GFP. The relief of quenching, as indicated by an increase in luminescence intensity upon addition of the clostridial toxin is indicative of the presence or activity of the clostridial toxin. These results further indicate that the use of gated emission can be useful for reducing background when assaying for clostridial toxin activity with a lanthanide-based substrate of the invention.

[023] Based on these findings, the present invention provides a clostridial toxin substrate which contains (a) a lanthanide donor complex; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (c) a clostridial toxin recognition sequence containing a cleavage site that intervenes between the lanthanide donor complex and the acceptor, where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor. In one embodiment, the invention provides a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence lifetime of at least 500 μ s. In another embodiment, the invention provides a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence quantum yield of at least 0.05. In still another embodiment, the invention provides a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence quantum yield of at least 0.5.

[024] Lanthanide ions useful in a lanthanide donor complex encompass, without limitation, terbium ions, europium ions, samarium ions and dysprosium ions. Lanthanide-binding sites useful in a lanthanide donor complex can have, for example, an affinity for a lanthanide ion of at least 5 μ M and include, but are not limited to, peptides and peptidomimetics such as, without limitation, those including the coordination site of an EF hand motif or including an EF hand motif. Lanthanide-binding sites useful in a lanthanide donor complex further include, yet are not limited to, thiol-reactive chelators; diethylenetriaminepentacetic acid (DTPA); β -diketone chelates; polyaminopolycarboxylic acid chelates; calixarene chelates; polyphenol; DOTA; pyridine and polypyridine. Additional lanthanide-binding sites useful in the invention include, without limitation, trisbipyridine (TBP) cryptates; trisbipyridine tetracarboxylate (TBP4COOH) cryptates; trisbipyridine pentacarboxylate (TBP5COOH) cryptates; and pyridine bipyridine tetracarboxylates (PBP4COOH).

[025] A lanthanide donor complex includes an antenna which can be distinct from, or incorporated within, the lanthanide-binding site of the donor complex. An antenna useful in the invention can be, without limitation, carbostyryl124 (CS124), tryptophan, or 2-hydroxyisophthalamide. In one embodiment, the invention provides a clostridial toxin substrate incorporating a lanthanide donor complex which includes carbostyryl124 (CS124) as the antenna. In another embodiment, the invention provides a clostridial toxin substrate in which the lanthanide donor complex is CS124-DTPA-EMCH-Tb.

[026] A variety of acceptors are useful in the clostridial toxin substrates of the invention including, without limitation, acceptor fluorophores such as Alexa Fluor dyes and other non-protein acceptors. Acceptor fluorophores useful in the invention further include, such as green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP). In one embodiment, the invention provides a clostridial toxin substrate which includes green fluorescent protein as the acceptor. Non-fluorescent acceptors also are useful in the clostridial toxin substrates of the invention and include, without limitation, heme proteins.

[027] A variety of recognition sequences can be included in a clostridial toxin substrate of the invention. In one embodiment, the recognition sequence is a BoNT/A recognition sequence such as, without limitation, a BoNT/A recognition sequence containing at least six consecutive residues of SNAP-25, where the six consecutive residues include Gln-Arg, or a peptidomimetic thereof. Such a BoNT/A recognition sequence can include, for example, residues 134 to 206 of SEQ ID NO: 2. A recognition sequence included in a clostridial toxin substrate of the invention also can be, without limitation, a BoNT/B recognition sequence. Such a BoNT/B recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof. In a further embodiment, a recognition sequence included in a clostridial toxin substrate is a BoNT/C1 recognition sequence. Such a BoNT/C1 recognition sequence can contain, without limitation, at least six consecutive residues of syntaxin, where the six consecutive residues include Lys-Ala, or a peptidomimetic thereof. A BoNT/C1 recognition sequence useful in the invention also can contain at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ala, or a peptidomimetic thereof.

[028] In a further embodiment, a recognition sequence included in a clostridial toxin substrate is a BoNT/D recognition sequence. Such a BoNT/D recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Lys-Leu, or a peptidomimetic thereof. A recognition sequence useful in the invention also can be, for example, a BoNT/E recognition sequence. Such a BoNT/E recognition sequence can contain, without limitation, at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ile, or a peptidomimetic thereof. In yet another embodiment, a recognition sequence included in a clostridial toxin substrate of the invention is a BoNT/F recognition sequence. BoNT/F recognition sequences useful in the invention encompass, without limitation, those having at least six consecutive residues of VAMP, where

the six consecutive residues include Gln-Lys, or a peptidomimetic thereof. A recognition sequence included in a clostridial toxin substrate also can be a BoNT/G recognition sequence. Such BoNT/G recognition sequences encompass, without limitation, those having at least six consecutive residues of VAMP, where the six consecutive residues include Ala-Ala, or a peptidomimetic thereof. In still a further embodiment, a recognition sequence included in a clostridial toxin substrate of the invention is a tetanus toxin (TeNT) recognition sequence. Such a TeNT recognition sequence can be, without limitation, a sequence containing at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof.

[029] A clostridial toxin substrate of the invention can be, without limitation peptide or peptidomimetic, which can have any of a variety of lengths. In particular embodiments, a clostridial toxin substrate of the invention is a peptide or peptidomimetic having at most 300 residues or at most 150 residues. A clostridial toxin substrate of the invention can be cleaved with a range of activities. In one embodiment, a clostridial toxin substrate of the invention can be cleaved with an activity of at least 1 nanomole/minute/milligram toxin. In another embodiment, a clostridial toxin substrate of the invention can be cleaved with an activity of at least 20 nanomoles/minute/milligram toxin. In a further embodiment, a clostridial toxin substrate of the invention can be cleaved with an activity of at least 100 nanomoles/minute/milligram toxin.

[030] The present invention further provides a nucleic acid molecule which contains a nucleotide sequence encoding a clostridial toxin substrate which includes (a), together with a lanthanide ion, a lanthanide donor complex; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (c) a clostridial toxin recognition sequence containing a cleavage site, where the cleavage site intervenes between the lanthanide donor complex and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor. A nucleic acid molecule of the invention can encode a clostridial toxin substrate with any of a variety of lengths; in particular embodiments, a nucleic acid molecule of the invention encodes a clostridial toxin substrate having a length of at most 300 residues, or a length of at most 150 residues.

[031] A lanthanide donor complex includes, in part, a lanthanide-binding site. Any of a variety of lanthanide-binding sites are useful in the invention including, without limitation, those which contain the coordination site of an EF hand motif and those which include an EF hand motif. In some embodiments, a lanthanide donor complex includes a tryptophan residues which acts as an antenna. In another embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate in which the acceptor is an acceptor fluorophore. In still further embodiments, a nucleic acid molecule of the invention encodes a clostridial toxin substrate in which the acceptor fluorophore is green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) or red fluorescent protein (RFP). In yet another embodiment, a nucleic acid molecule of the invention encodes a

clostridial toxin substrate in which the acceptor is a non-fluorescent acceptor such as, without limitation, a heme protein.

[032] A clostridial toxin substrate encoded by a nucleic acid molecule of the invention can include any of a variety of recognition sequences. In a nucleic acid molecule of the invention, the encoded recognition sequence can be, for example, a BoNT/A recognition sequence such as, without limitation, a BoNT/A recognition sequence containing at least six consecutive residues of SNAP-25, where the six consecutive residues include Gln-Arg, or a peptidomimetic thereof. Such a BoNT/A recognition sequence can include, for example, residues 134 to 206 of SEQ ID NO: 2. An encoded recognition sequence useful in a nucleic acid molecule of the invention also can be, without limitation, a BoNT/B recognition sequence. Such a BoNT/B recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof. In a further embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate which includes a BoNT/C1 recognition sequence. Such a BoNT/C1 recognition sequence can contain, without limitation, at least six consecutive residues of syntaxin, where the six consecutive residues include Lys-Ala, or a peptidomimetic thereof. A BoNT/C1 recognition sequence useful in the invention also can contain at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ala, or a peptidomimetic thereof.

[033] In a further embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate which includes a BoNT/D recognition sequence. Such a BoNT/D recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Lys-Leu, or a peptidomimetic thereof. In another embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate which includes a BoNT/E recognition sequence. Such a BoNT/E recognition sequence can contain, without limitation, at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ile, or a peptidomimetic thereof. In yet another embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate which includes a BoNT/F recognition sequence. BoNT/F recognition sequences useful in the invention encompass, without limitation, those having at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Lys, or a peptidomimetic thereof. A nucleic acid molecule of the invention also can encode a clostridial toxin substrate which has a BoNT/G recognition sequence. Such a BoNT/G recognition sequence can be, for example, one having at least six consecutive residues of VAMP, where the six consecutive residues include Ala-Ala, or a peptidomimetic thereof. In another embodiment, a nucleic acid molecule of the invention encodes a clostridial toxin substrate which includes a TeNT recognition sequence. Such a TeNT recognition sequence can be, without limitation, a sequence containing at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof.

[034] The tetanus and botulinum neurotoxins which can be assayed using a substrate or method of the invention are produced by *Clostridia*. These toxins cause the neuromuscular syndromes of tetanus and botulism, with tetanus toxin acting mainly within the central nervous system and botulinum toxin acting on the peripheral nervous system. Clostridial neurotoxins share a similar mechanism of cell intoxication in which the release of neurotransmitters is blocked. In these toxins, which are composed of two disulfide-linked polypeptide chains, the larger subunit is responsible for neurospecific binding and translocation of the smaller subunit into the cytoplasm. Upon translocation and reduction in neurons, the smaller chain displays peptidase activity specific for protein components involved in neuroexocytosis. 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.

[035] Tetanus neurotoxin and botulinum neurotoxins B, D, F, and G specifically recognize VAMP (also known as synaptobrevin), an integral protein of the synaptic vesicle membrane. VAMP is cleaved at distinct bonds depending on the neurotoxin. Botulinum A and E neurotoxins recognize and cleave specifically SNAP-25, a protein of the presynaptic membrane, at two different sites in the carboxy-terminal portion of the protein. Botulinum neurotoxin C cleaves syntaxin, a protein of the nerve plasmalemma, in addition to SNAP-25. The three protein targets of the Clostridial neurotoxins are conserved from yeast to humans although cleavage sites and toxin susceptibility are not necessarily conserved (see below; see, also, Humeau et al., *Biochimie* 82:427-446 (2000); Niemann et al., *Trends in Cell Biol.* 4:179-185 (1994); and Pellizzari et al., *Phil. Trans. R. Soc. London* 354:259-268 (1999)).

[036] Naturally occurring tetanus and botulinum neurotoxins are produced as polypeptide chains of 150 kDa without a leader sequence. These toxins may be cleaved by bacterial or tissue proteinases at an exposed protease-sensitive loop, generating dichain toxin. Selective proteolytic cleavage activates the toxins by generating two disulfide-linked chains: an L chain of 50 kDa and an H chain of 100 kDa, which is composed of two domains denoted H_N and H_C. This dichain toxin is substantially more active than the unnicked toxin. Naturally occurring clostridial toxins contain a single interchain disulfide bond bridging the heavy chain and light chain; such a bridge is important for neurotoxicity of toxin added extracellularly (Montecucco and Schiavo, *Quarterly Rev. Biophysics* 28:423-472 (1995)).

[037] The clostridial toxins appear to be folded into three distinct domains of about 50 kDa which are connected by loops, with each domain having a distinct functional role. As illustrated in Figure 1, the cell intoxication mechanism of the clostridial toxins consists of four distinct steps: (1) binding; (2) internalization; (3) membrane translocation; and (4) enzymatic target modification. The carboxy-terminal domain of the heavy chain (H_C) functions in neurospecific binding, while the amino-terminal domain of the H chain (H_N) functions in membrane translocation from endosome to cell cytoplasm. Following reduction of the disulfide linkage inside the cell, the zinc-endopeptidase activity of the L chain is liberated (Montecucco and Schiavo, *supra*, 1995).

[038] The amino acid sequences of eight human clostridial neurotoxin serotypes have been derived from the corresponding genes (Niemann, "Molecular Biology of Clostridial Neurotoxins" in Sourcebook of Bacterial Protein Toxins Alouf and Freer (Eds.) pp. 303-348 London: Academic Press 1991). The L chain and H chain are composed of roughly 439 and 843 residues, respectively. Homologous segments are separated by regions of little or no similarity. The most well conserved regions of the L chain are the amino-terminal portion (100 residues) and central region (corresponding to residues 216 to 244 of TeNT), as well as the two cysteines forming the interchain disulfide bond. The 216 to 244 region contains a His-Glu-X-X-His binding motif characteristic of zinc-endopeptidases.

[039] The clostridial toxin heavy chains are less well conserved than the light chains, with the carboxy-terminal portion of H_C corresponding to residues 1140 to 1315 of TeNT the most variable. This is consistent with the involvement of the H_C domain in binding to nerve terminals and the fact that different neurotoxins appear to bind different receptors.

[040] Comparison of the nucleotide and amino acid sequences of the clostridial toxins indicates that they derive from a common ancestral gene. Spreading of these genes may have been facilitated by the fact that the clostridial neurotoxin genes are located on mobile genetic elements. As discussed further below, sequence variants of the seven botulinum toxins are known in the art. See, for example, Humeau et al., *supra*, 2000.

[041] As discussed above, natural targets of the clostridial neurotoxins include VAMP, SNAP-25, and syntaxin. VAMP is associated with the synaptic vesicle membrane, whereas SNAP-25 and syntaxin are associated with the target membrane (see Figure 2). BoNT/A and BoNT/E cleave SNAP-25 in the carboxy-terminal region, releasing nine or twenty-six amino acid residues, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxy-terminus. 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. Thus, BoNT/B, BoNT/C1, BoNT/D, BoNT/F, BoNT/G or TeNT proteolysis results in release of a large portion of the cytosolic domain of VAMP or syntaxin, while only a small portion of SNAP-25 is released by BoNT/A, BoNT/C1 or BoNT/E cleavage (Montecucco and Schiavo, *supra*, 1995).

[042] Naturally occurring SNAP-25, a protein of about 206 residues lacking a transmembrane segment, is associated with the cytosolic surface of the nerve plasmalemma (Figure 2; see, also, Hodel et al., Int. J. Biochemistry and Cell Biology 30:1069-1073 (1998)). In addition to homologs highly conserved from *Drosophila* to mammals, SNAP-25-related proteins also have been cloned from yeast. SNAP-25 is required for axonal growth during development and may be required for nerve terminal plasticity in the mature nervous system. In humans, two isoforms are differentially expressed during development; isoform a is constitutively expressed during fetal development, while isoform b appears at birth and

predominates in adult life. SNAP-25 analogues such as SNAP-23 also are expressed outside the nervous system, for example, in pancreatic cells.

[043] Naturally occurring VAMP is a protein of about 120 residues, with the exact length depending on the species and isotype. As shown in Figure 2, VAMP contains a short carboxy-terminal segment inside the vesicle lumen while most of the molecule is exposed to the cytosol. The proline-rich amino-terminal thirty residues are divergent among species and isoforms while the central portion of VAMP (residues 30 to 96), which is rich in charged and hydrophilic residues and includes known cleavage sites, is highly conserved. VAMP colocalizes with synaptophysin on synaptic vesicle membranes.

[044] A variety of species homologs of VAMP are known in the art including human, rat, bovine, *Torpedo*, *Drosophila*, yeast, squid and *Aplysia* homologs. In addition, multiple isoforms of VAMP have been identified including VAMP-1, VAMP-2 and cellubrevin, and forms insensitive to toxin cleavage have been identified in non-neuronal cells. VAMP appears to be present in all vertebrate tissues although the distribution of VAMP-1 and VAMP-2 varies in different cell types. Chicken and rat VAMP-1 are not cleaved by TeNT or BoNT/B. These VAMP-1 homologs have a valine in place of the glutamine present in human and mouse VAMP-1 at the TeNT or BoNT/B cleavage site. The substitution does not affect BoNT/D, /F or /G, which cleave both VAMP-1 and VAMP-2 with similar rates.

[045] Syntaxin is located on the cytosolic surface of the nerve plasmalemma and is membrane-anchored via a carboxy-terminal segment, with most of the protein exposed to the cytosol. Syntaxin colocalizes with calcium channels at the active zones of the presynaptic membrane, where neurotransmitter release takes place. In addition, syntaxin interacts with synaptotagmin, a protein of the SSV membrane, that forms a functional bridge between the plasmalemma and the vesicles. A variety of syntaxin isoforms have been identified. Two isoforms of slightly different length (285 and 288 residues) have been identified in nerve cells (isoforms 1A and 1B), with isoforms 2, 3, 4 and 5 expressed in other tissues. The different isoforms have varying sensitivities to BoNT/C1, with the 1A, 1B, 2 and 3 syntaxin isoforms cleaved by this toxin.

[046] The lanthanides, or "rare earth" elements, are a group of elements whose trivalent cations emit light at well-defined wavelengths and with long decay times. Lanthanides include, without limitation, elements with atomic numbers 57 through 71: lanthanide (La); cerium (Ce); praseodymium (Pr); neodymium (Nd); promethium (Pm); samarium (Sm); europium (Eu); gadolinium (Gd); terbium (Tb); dysprosium (Dy); holmium (Ho); erbium (Er); thulium (Tm); ytterbium (Yb); and lutetium (Lu). Lanthanides can further include, without limitation, yttrium (Y; atomic number 39) and scandium (Sc; atomic number 21).

[047] Lanthanide ions have unique photophysical and spectral properties based on their special electronic configuration which partly shields optically active electrons. The emission lifetimes of the

lanthanide ions are usually long; however, their light collection efficiency is very poor. Given these properties, lanthanide ions are particularly useful in conjunction with a light-harvesting device ("antenna"), which can be, for example, a strongly absorbing aromatic chromophore such as a pyridyl, phenyl or indole group. The energy collected by the antenna is transferred by intramolecular non-radiative processes from the singlet to the triplet state of the moiety, then from the triplet to the emissive level of the lanthanide ion, which subsequently emits its characteristic long-lived luminescence. Thus, a lanthanide ion in conjunction with an antenna is useful as a luminescent probe, for example, in highly sensitive time-resolved assays, where it generates a long-lived fluorescent signal that can be readily distinguished from short-lived background fluorescence present in many biological samples.

[048] Lanthanides generally exist as trivalent cations, in which case their electronic configuration is $(\text{Xe})4f^n$, with n varying from 1 (Ce^{3+}) to 14 (Lu^{3+}). Without wishing to be bound by the following, the transitions of the f-electrons can be responsible for the special photophysical properties of the lanthanide ions such as long-lived luminescence and sharp absorption and emission lines. In particular, f-electrons can be shielded from external perturbations by filled 5s and 5p orbitals, resulting in characteristic line-like spectra. f-f electronic transitions are forbidden, leading to long excited state lifetimes in the microsecond to millisecond range.

[049] As discussed above, in many cases energy can be transferred to a lanthanide ion from a nearby organic chromophore, known as an "antenna" or "sensitizer." Thus, a lanthanide donor complex useful in the invention includes a lanthanide ion, a lanthanide-binding site and an antenna and generally is structured to shield the lanthanide ion from the quenching effects of water or other solvent. The lanthanide-binding site functions to retain the lanthanide ion and may optionally act as a scaffold for attachment of an antenna and a reactive group suitable for coupling the lanthanide donor complex to the remainder of the clostridial toxin substrate. In one embodiment, the antenna is incorporated within the lanthanide-binding site. In another embodiment, an antenna separate from the lanthanide-binding site is included in the lanthanide donor complex.

[050] Lanthanide ions useful in the invention include, without limitation, terbium (Tb), europium (Eu), dysprosium (Dy) and samarium (Sm) ions, which are lanthanides that emit in the visible spectra. In one embodiment, a lanthanide ion is a Tb or Eu ion, which has a high emission quantum yield and emits with stronger intensity than a Dy or Sm ion. Excitation of an antenna for Tb or Eu is in the ultraviolet range and can be achieved, for example, using a nitrogen laser at 337 nm, or a flash lamp. Terbium emission is in the green spectra, while europium emission is in the red spectra, both providing a contrast to the excitation light.

[051] As used herein, the term "antenna" is synonymous with "sensitizer" and means a molecule such as an organic chromophore which absorbs excitation light and transfers the light energy to a lanthanide ion. An antenna is necessary because of the inherently weak absorbance of lanthanide ions themselves.

In one embodiment, the antenna is carbostyryl124 (CS124), which absorbs light with an excitation of 337 nm. In another embodiment, the antenna is a tryptophan residue. In a further embodiment, the antenna is 2-hydroxyisophthalamide, which also acts as a lanthanide-binding site (see below). It is understood that an antenna can be distinct from, or can make up part of a lanthanide binding-site. As non-limiting examples, an antenna which binds a lanthanide ion can be 2-hydroxyisophthalamide, a pyridine or other cryptate; a LANCE complex (Wallac; Perkin-Elmer); or a terpyridine complex.

[052] As used herein, the term "lanthanide-binding site" means a moiety that constrains a lanthanide ion. A variety of lanthanide-binding sites are useful in the clostridial toxin substrates of the invention. Exemplary classes of lanthanide-binding sites include, but are not limited to, polyaminopolycarboxylic acid chelates such as DTPA chelates, BPTA chelates, β -diketone chelates, pyridines, polypyridines and calixarene chelates. These and other lanthanide chelates are known in the art as described in Li and Selvin, Bioconj. Chem. 8:127-132 (1997); Chen and Selvin, Bioconj. Chem. 10: 311-315 (1999); Selvin, Nature Struc. Biol. 7:730-734 (2000); Selvin, Methods Enzym. 246:300-334 (1995); Selvin et al., J. Am. Chem. Soc. 116:6029-6030 (1994); and Yuan et al., Anal. Chem. 73:1869-1876 (2001). In one embodiment, a lanthanide-binding site useful in the invention is a polyaminocarboxylate such as diethylenetriaminepentacetic acid (DTPA) or triethylenetetraaminehexaacetic acid (TTHA). An antenna which is useful in conjunction with a polyaminocarboxylate lanthanide-binding site such as DTPA or TTHA can be, without limitation, carbostyryl124 (CS124).

[053] Lanthanide-binding sites useful a lanthanide donor complex include those which are peptides and peptidomimetics. In one embodiment, a lanthanide-binding site useful in the invention includes the coordination site of an EF hand motif, which is a highly conserved domain in which two helices enclose a binding loop with high affinity for Ca^{2+} , Tb^{3+} and other ions with similar ionic radii. In nature, more than 200 proteins including calmodulin, troponin C, parvalbumin and calbindin contain one or several copies of an EF hand.

[054] In nature, the two α -helices of an EF hand motif are connected by a loop of about 12 residues which contains the metal coordination site of the motif. The residues which serve as ligands are highly conserved within a contiguous sequence of twelve residues spanning the loop and the beginning of the second helix. In particular, the residues at positions 1, 3, 5, 7, 9, and 12 of this loop region and possibly a coordinating water molecule provide seven coordination oxygens for the lanthanide ion. Acidic amino acids are frequently present at most or all of the coordinating positions with the exception of Trp at position 7, where the coordination oxygen is provided by the main chain (Vasquez-Ibar et al., Proc. Natl. Acad. Sci. USA 99:3487-3492 (2002)). Loop residues in positions 1, 3, 5 and 12 contribute monodentate (positions 1, 3 and 5) or bidentate (position 12) ligands through side chain oxygens; residue 7 (tryptophan) ligands through its backbone carbonyl oxygen. An invariant glycine residue is present at position 6 to allow the sharp bend necessary to ligate the lanthanide through the oxygen of residue 5 and the carbonyl of residue 7. In addition, residue 9 provides a ligand either directly through an oxygen of its

side chain or indirectly via a water molecule. Residue 12 is an invariant glutamic acid (Glu), while residue 1 is typically aspartate (Asp). See Lewit-Bentley, Curr. Opin. Struct. Biol. 10:637-643 (2000); and Myers (Ed.), Molecular Biology and Biotechnology VCH publishers New York, NY (1995).

[055] As used herein, the term "coordination site of an EF hand motif" means a sequence of about 12 residues in which position 6 is a glycine; position 12 is a glutamic acid, and ligand groups at positions 1, 3, 5, 7, 9, and 12 of the sequence, or a coordinating water molecule, provide a metal binding site. A tryptophan residue optionally can be present at position 7. It is understood that a lanthanide-binding site which includes the coordination site of an EF hand motif may or may not have homology to the α -helices of an EF hand motif outside the 12 residue coordination site.

[056] A sequence which includes the coordination site of an EF hand motif can be, for example, the 14-mer peptide GDKNADGWIEFEEL (SEQ ID NO: 97) as described in MacManus et al., Biosci. Rep. 3:1071-1075 (1983), and Strynadka and James, Annu. Rev. Biochem. 58: 951-998 (1989). The 14-mer SEQ ID NO: 97 functions as both a lanthanide-binding site and an antenna due to the inclusion of a tryptophan residue. Coordination sites of an EF hand motif further include, without limitation, the peptide GDKNADGFI~~C~~FEEL (SEQ ID NO: 98), where the indicated cysteine residue can be covalently labeled with iodoacetamidosalicylic acid or another antenna (Clark et al., FEBS 333: 96-98 (1993)), and the peptide DKNADG~~C~~IEFEE (SEQ ID NO: 99), where the indicated cysteine residue permits convenient covalent attachment of an antenna (Clark et al., Anal. Biochem. 210:1-6 (1993)). As non-limiting examples, 7-diethylamino-3-((4'-iodoacetylaminophenyl)-4-methylcoumarin can be covalently attached to the cysteine in SEQ ID NO: 99, for example, as an antenna for Eu^{3+} , and 4-iodoacetamidosalicylic acid can be covalently attached to the cysteine in SEQ ID NO: 99, for example, as an antenna for Tb^{3+} .

[057] A lanthanide-binding site which includes the coordination site of an EF hand motif also can be a lanthanide-binding tag (LBT) such as one described in Nitz et al., Angew. Chem. Int. Ed. 43:3682-3685 (2004)). Such a lanthanide-binding site can include, without limitation, the 17-mer YID₁TN₃ND₅GW₇YE₉GDE₁₂LLA (SEQ ID NO: 100), which includes the antenna tryptophan. Such a lanthanide-binding site can, for example, coordinate a terbium or other lanthanide ion through eight ligands, in particular, monodentate oxygen ligands of Asp1, Asn3 and Asp5, bidentate ligands from Glu9 and Glu12, and the backbone carbonyl of Trp 7. Furthermore, lanthanide-binding sites such as those described in Nitz et al., *supra*, 2004, can bind a terbium or other lanthanide ion with nanomolar affinities. As non-limiting examples, the lanthanide-binding site SEQ ID NO: 100 binds Eu^{3+} with an apparent dissociation constant K_d of 62 +/- 4 nM; Gd^{3+} with an apparent dissociation constant K_d of 84 +/- 6 nM; Tb^{3+} with an apparent dissociation constant K_d of 57 +/- 3 nM; Dy^{3+} with an apparent dissociation constant K_d of 71 +/- 5 nM; and Er^{3+} with an apparent dissociation constant K_d of 78 +/- 6 nM.

[058] Lanthanide-binding sites useful in a lanthanide donor complex further include those which bind a lanthanide ion exclusively through peptide-based ligands, excluding water molecules from the lanthanide

ion coordination sphere. Such a lanthanide-binding site can include, for example, the 17-mer sequence YIDTNN DGWYEGDELLA (SEQ ID NO: 100; Nitz et al., *supra*, 2004).

[059] A lanthanide-binding site useful in a lanthanide donor complex also can be an EF hand motif. As used herein, the term "EF hand motif" means two α -helices flanking the coordination site of an EF hand motif. An EF hand motif useful in the invention can be, without limitation, an EF hand from one of the following subfamilies: calmodulin (CAM); troponin C (TNC); essential or regulatory light chain of myosin; troponin, nonvertebrate (TPNV); Cal1, *C. elegans* (CAL); squidulin, *Loligo* (SQUID); CDC31 and caltractin (CDC); calcium-dependent protein kinase (CDPK); LAV1, *Physarum* (LAV); EHF5; calcineurin B (CLNB); p24 thyroid protein, *Canis* (TPP); calbindin 28 kDa (CLBN); parvalbumin (PARV); intestinal calcium binding protein and S100; diacylglycerol kinase (DGK); α -actinin (ACTN); protein phosphatase, *Drosophila* (PTTS); *Strongylocentrotus* calcium-binding protein (SPEC); *Lytechinus purpuratus* SPEC resembling protein (LPS); Aequorin and luciferin binding protein (AEQ); calcium vector protein, *Branchiostoma* (CVP); 1F8 and TB17 (1F8); calpain and sorcin (CALP); surface protein, *Plasmodium* (PFS); sarcoplasm calcium-binding protein (SARC); visinin and recoverin (VIS); calcium-binding protein, *Saccharopolyspora* (CMSE); *Tetrahymena* calcium-binding protein (TCBP); CAM related gene product, *Homo* (CRGP); or protein kinase, *Plasmodium* (PFPK). An EF hand motif useful in the invention also can be a canonical EF hand motif as shown in Figure 6 or a peptide having significant amino acid homology to a naturally occurring EF hand, for example, at least 60%, 70%, 80%, 90% or 95% amino acid identity with a naturally occurring EF hand such as a member of one of the subfamilies described above. A variety of naturally occurring EF hands are known in the art, as described, for example, in Kawasaki and Kretsinger, Protein Profile 1:343-517 (1994), and Nakayama and Kretsinger, Annu. Rev. Biophys. Biomol. Struct. 23:473-507 (1994). Furthermore, methods of genetically engineering an EF hand motif or the coordination site of an EF hand motif also are well known in the art. See, for example, Vazquez-Ibar et al., Proc. Natl. Acad. Sci. USA 99:3487-3492 (2002).

[060] Lanthanide-binding sites useful in a lanthanide donor complex further include chimeric helix-turn-helix/EF hand peptides, which are helix-turn-helix DNA binding motifs redesigned to include a lanthanide binding site. Such lanthanide-binding sites include, without limitation, the peptide "P3W" (TERRQQLDKDGDTIDEREIKIWFQNKRAKIK; SEQ ID NO: 101) as described in Welch et al., Proc. Natl. Acad. Sci. USA 100:3725-3730 (2003).

[061] Additional peptide lanthanide-binding sites are known in the art and include, yet are not limited to, those in which the lanthanide-binding site appears to be adventitious or is an intrinsic calcium-binding site. As non-limiting examples, lanthanide ions bind strongly to *Bacillus subtilis* PyrR (Tomchick et al., Structure 6:337-350 (1998)) and the cadherin NCD1 (Moore et al., J. Am. Chem. Soc. 120:7105-7106 (1998)). See, also, Pidcock and Moore, J. Biol. Inorg. Chem. 6:479-489 (2001). Peptide lanthanide-binding sites also include those identified using screening protocols based, for example, on terbium

luminescence (Franz et al., Chem. BioChem. 4:265 (2003); and Nitz et al., Chem. BioChem. 4:272 (2003)) and those identified using similar screening assays.

[062] A lanthanide-binding site useful in a lanthanide donor complex also can be a cryptate, which is a macropolycyclic compound that acts as a cage, trapping a lanthanide ion and protecting it from solvent. The cryptate cage itself acts as an antenna for the trapped lanthanide ion, specifically by absorbing excitation light and transferring the energy to the ion and by protecting it from quenching by water. A variety of lanthanide cryptates are useful in the invention including, but not limited to, trisbipyridine (TBP) lanthanide cryptates and derivatives thereof. Such cryptates, which are tightly associated with their ions, are highly stable in biological media. Lanthanide cryptates useful in the invention include, without limitation, trisbipyridine europium cryptates; trisbipyridine tetracarboxylate (TBP4COOH) europium cryptates; trisbipyridine pentacarboxylate europium cryptates and pyridine bipyridine tetracarboxylate (PBP4COOH) europium cryptates. One skilled in the art understands that cryptate derivatives containing multiple carboxylic groups such as TBP4COOH or PBP4COOH can be significantly more luminescent than their parent cryptate. These and other lanthanide cryptates are well known in the art, as described, for example, in Selvin et al., Ann. Rev. Biomol. Struct. 31:275-302 (2002); Mathis, Clin. Chem. 41:1391-1397 (1995); and Mathis, J. Clin. Ligand Assay 20:141-147 (1997).

[063] Lanthanide-binding sites useful in a lanthanide donor complex further include 2-hydroxyisophthalamide, a molecule which forms luminescent and highly stable complexes with lanthanides such as Sm^{3+} , Eu^{3+} , Tb^{3+} and Dy^{3+} (Petoud et al., J. Am. Chem. Soc. 125:13324-13325 (2003)). The 2-hydroxyisophthalamide group is a very good ligand for lanthanide ions, providing, for example, excellent sensitization of Tb^{3+} through a particularly efficient ligand-to-lanthanide energy transfer process. The quantum yields of 2-hydroxyisophthalamide lanthanide chelates can be quite high ($\Phi > 0.5$), and complexes formed with 2-hydroxyisophthalamides are generally highly soluble and stable in water at physiological pH (Petoud et al., *supra*, 2003).

[064] A lanthanide-binding site useful in a lanthanide donor complex also can be a β -diketonate such as, without limitation, a Eu^{3+} - β -diketonate (2-naphthoyltrifluoroacetate)-trioctylphosphine oxide ternary fluorescent complex. Such lanthanide-binding sites are well known in the art as described, for example, in Diamandis, Clin. Biochem. 21:139-150 (1988), and are commercially available, for example, as part of the DELFIA[®] system (Perkin-Elmer).

[065] One skilled in the art understands that these and other lanthanide-binding sites can be useful as part of a lanthanide donor complex in the clostridial toxin substrates and methods of the invention. Such lanthanide-binding sites encompass, but are not limited to, those containing 4,7-bis(chlorosulfodiphenyl)-1,10, phenanthroline-2,9-dicarboxylic acid ("FIAGEN" system; Diamandis et al., Anal. Chem. 62:1149A-1157A (1990)) and those containing 5-fluorosalicylate- Tb^{3+} -EDTA ("enzyme-amplified time-resolved fluoroimmunoassay" system; Chrisopoulos and Diamandis, Anal. Chem. 64:342-346 (1992)). See, also;

Cooper and Sammes, J. Chem. Soc. Perkin Trans. 28:1675-1700; Jones et al., J. Fluoresc. 11:13-21 (2001); and Kolb et al. in Devlin (Ed.), High Throughput Screening: The Discovery of Bioactive Substances pages 345-360 New York: Marcel Dekker (1997)). One skilled in the art understands that these and other peptide, peptidomimetic and small molecule lanthanide-binding sites can be incorporated into a lanthanide donor complex in a substrate of the invention.

[066] Lanthanide-binding sites useful in a lanthanide donor complex further include, without limitation, those with an affinity for a lanthanide ion in the nanomolar to picomolar range. In particular embodiments, a lanthanide-binding site useful in the invention has K_d for a lanthanide ion of less than 10 μM , less than 5 μM , less than 1 μM , less than 500 nM, less than 250 nM, less than 100 nM, less than 50 nM, less than 10 nM, less than 1 nM or less than 0.1 nM. In further embodiments, a lanthanide-binding site useful in the invention has K_d for a lanthanide ion of less than 100 nM, less than 90 nM, less than 80 nM, less than 70 nM, less than 60 nM, less than 50 nM, less than 40 nM, less than 30 nM, less than 20 nM, or less than 10 nM. In still further embodiments, a lanthanide-binding site useful in the invention has K_d for a lanthanide ion of less than 1×10^{-9} M, less than 1×10^{-10} M, less than 1×10^{-11} M, less than 1×10^{-12} M, less than 1×10^{-13} M, less than 1×10^{-14} M, less than 1×10^{-15} M, less than 1×10^{-16} M, less than 1×10^{-17} M, less than 1×10^{-18} M, less than 1×10^{-19} M or less than 1×10^{-20} M.

[067] As used herein, the term “acceptor” means a molecule that can absorb energy from, and upon excitation of, a lanthanide donor complex. An acceptor useful in a clostridial toxin substrate has an absorbance spectrum which overlaps the emission spectrum of the lanthanide donor complex included in the substrate. An acceptor useful in the invention generally has rather low absorption at a wavelength suitable for excitation of the antenna incorporated in the lanthanide donor complex.

[068] As set forth above, an acceptor has an absorbance spectrum that overlaps the emission spectrum of the lanthanide donor complex. The term “overlapping,” as used herein in reference to the absorbance spectrum of an acceptor and the emission spectrum of a lanthanide donor complex, means an absorbance spectrum and emission spectrum that are partly or entirely shared. Thus, in such overlapping spectra, the high end of the range of the emission spectrum of the lanthanide donor complex is higher than the low end of the range of the absorbance spectrum of the acceptor.

[069] A clostridial toxin substrate useful in the invention contains a cleavage site that “intervenes” between a lanthanide donor complex and an acceptor. Thus, the cleavage site is positioned in between the lanthanide donor complex and the acceptor such that proteolysis at the cleavage site results in a first cleavage product containing the lanthanide donor complex and a second cleavage product containing the acceptor. It is understood that all or only a portion of the clostridial toxin recognition sequence may intervene between the lanthanide donor complex and the acceptor.

[070] A clostridial toxin substrate useful in the invention also contains a clostridial toxin recognition sequence which includes a cleavage site. By definition, a clostridial toxin substrate is susceptible to cleavage by at least one clostridial toxin under conditions suitable for clostridial toxin protease activity.

[071] As used herein, the term "clostridial toxin recognition sequence" means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a clostridial toxin under conditions suitable for clostridial toxin protease activity. A variety of clostridial toxin recognition sequences are discussed hereinbelow.

[072] In particular embodiments, a clostridial toxin substrate useful in the invention is a peptide or peptidomimetic having a defined length. A clostridial toxin substrate can be, for example, a peptide or peptidomimetic having at least 100, at least 150, at least 200, at least 250, at least 300, at least 350 or at least 500 residues. In other embodiments, a clostridial toxin substrate has at most 20 residues, at most 30 residues, at most 40 residues, at most 50 residues, at most 100 residues, at most 150 residues, at most 200 residues, at most 250 residues, at most 300 residues, at most 350 residues or at most 400 residues.

[073] It is understood that a clostridial toxin substrate useful in the invention optionally can include one or more additional components. As a non-limiting example, a flexible spacer sequence such as GGGGS (SEQ ID NO: 21) can be included in a clostridial toxin substrate useful in the invention. A useful clostridial toxin substrate further can include, without limitation, one or more of the following: an affinity tag such as HIS6; biotin or a biotinylation sequence; an epitope such as FLAG, hemagglutinin (HA), c-myc, or AU1; an immunoglobulin hinge region; an N-hydroxysuccinimide linker; a peptide or peptidomimetic hairpin turn; or a hydrophilic sequence or another component or sequence that, for example, facilitates purification or promotes the solubility or stability of the clostridial toxin substrate.

[074] A clostridial toxin substrate of the invention contains a lanthanide donor complex and an acceptor, where the clostridial toxin cleavage site is positioned between the lanthanide donor complex and acceptor. In one embodiment, the acceptor is positioned carboxy-terminal of the cleavage site while the lanthanide donor complex is positioned amino-terminal of the cleavage site. In another embodiment, the acceptor is positioned amino-terminal of the cleavage site while the lanthanide donor complex is positioned carboxy-terminal of the cleavage site.

[075] Substrates useful in the invention can be prepared by recombinant methods or using synthetic chemical methods, or a combination thereof. As described herein in Example I, a fusion protein containing GFP fused to a BoNT/A clostridial toxin recognition sequence and a carboxy-terminal cysteine was prepared by recombinant methods. The carboxy-terminal cysteine was used for attachment of a lanthanide donor complex to produce the complete clostridial toxin substrate. Recombinant methods for preparation of clostridial toxin substrates which are fusion proteins are well known in the art as described,

for example, in Ausubel, Current Protocols in Molecular Biology John Wiley & Sons, Inc., New York 2000.

[076] Routine chemical methods suitable for modifying a protein, peptide or peptidomimetic to contain a lanthanide donor complex or acceptor or both are well known in the art (Fairclough and Cantor, Methods Enzymol. 48:347-379 (1978); Glaser et al., Chemical Modification of Proteins Elsevier Biochemical Press, Amsterdam (1975); Haugland, Excited States of Biopolymers (Steiner Ed.) pp. 29-58, Plenum Press, New York (1983); Means and Feeney, Bioconjugate Chem. 1:2-12 (1990); Matthews et al., Methods Enzymol. 208:468-496 (1991); Lundblad, Chemical Reagents for Protein Modification 2nd Ed., CRC Press, Boca Raton, Florida (1991); Haugland, *supra*, 1996). As non-limiting examples, a lanthanide donor complex can include an amine-reactive group such as an isothiocyanate (Li and Selvin, Bioconj. Chem. 8:127-132 (1997) or a thiol-reactive group such as a maleimide, bromoacetamide or pyridyl dithio (Chen and Selvin, Bioconjug. Chem. 10:311-315 (1999)). A thiol-reactive lanthanide donor complex is conveniently attached, for example, to a cysteine residue in the substrate. Where a portion of the clostridial toxin substrate is prepared using recombinant techniques, it is understood that a cysteine residue can be engineered at the appropriate position of the substrate for attachment of the lanthanide donor complex (see Example I). Haloacetyl labeling reagents also can be used to couple a lanthanide donor complex or acceptor in preparing a clostridial toxin substrate useful in the invention. See, for example, Wu and Brand, *supra*, 1994.

[077] Cross-linker moieties also can be useful for preparing a clostridial toxin substrate of the invention. Cross-linkers are well known in the art and include homo- and hetero-bifunctional cross-linkers such as BMH and SPDP. Where the lanthanide-binding site or acceptor is a protein, well known chemical methods for specifically linking molecules to the amino- or carboxy-terminus of a protein can be employed. See, for example, "Chemical Approaches to Protein Engineering" in Protein Engineering: A Practical Approach Rees et al. (Eds) Oxford University Press, 1992.

[078] Lanthanide atoms and DTPA and TTHA chelates are available from a variety of commercial sources including Invitrogen and Sigma. Furthermore, synthesis and purification of DTPA-CS124 and TTHA-CS124 can be routinely performed, for example, as described in Li and Selvin, J. Am. Chem. Soc. 117:8132 (1995). Trisbipyridine (TBP) and tetracarboxylate (TBP4COOH) europium cryptates are commercially available, for example, from CIS Bio International (Bedford, MA) or can be prepared by routine methods. One skilled in the art understands that these and other routine recombinant and synthetic chemical methods can be used to prepare a clostridial toxin substrate useful in the invention.

[079] Further provided herein is a method of determining the presence or activity of a clostridial toxin by (a) treating with a sample, under conditions suitable for clostridial toxin protease activity, a clostridial toxin substrate containing (i) a lanthanide donor complex; (ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of the lanthanide donor complex; and (iii) a clostridial toxin recognition

sequence containing a cleavage site that intervenes between the lanthanide donor complex and the acceptor, where, under the appropriate conditions, resonance energy transfer is exhibited between the lanthanide donor complex and the acceptor; (b) exciting an antenna of said lanthanide donor complex; and (c) determining resonance energy transfer of the treated substrate relative to a control substrate, where a difference in resonance energy transfer of the treated substrate as compared to the control substrate is indicative of the presence or activity of the clostridial toxin. In one embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence lifetime of at least 500 μ s. In another embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence quantum yield of at least 0.05. In still another embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a lanthanide donor complex having a fluorescence quantum yield of at least 0.5.

[080] A lanthanide donor complex includes a lanthanide ion such as, without limitation, a terbium ion, europium ion, samarium ion or dysprosium ion. Lanthanide-binding sites useful a lanthanide donor complex encompass, but are not limited to, those having an affinity for a lanthanide ion of at least 5 μ M, including, without limitation, peptides and peptidomimetics such as those including the coordination site of an EF hand motif or including an EF hand motif. A lanthanide-binding site useful in a lanthanide donor complex can be, without limitation, a thiol-reactive chelator; diethylenetriaminepentacetic acid (DTPA); β -diketone chelate; polyaminopolycarboxylic acid chelate; calixarene chelate; polyphenol; DOTA; pyridine; polypyridine; trisbipyridine (TBP) cryptate; trisbipyridine tetracarboxylate (TBP4COOH) cryptate; trisbipyridine pentacarboxylate (TBP5COOH) cryptate; or pyridine bipyridine tetracarboxylate (PBP4COOH).

[081] In a method of the invention, the lanthanide donor complex includes an antenna, which can be separate from, or incorporated within, the lanthanide-binding site. Thus, a method of the invention can be practiced, for example, with an antenna which is carbostyryl124 (CS124), tryptophan, or 2-hydroxyisophthalamide. In one embodiment, a method of the invention is practiced with a clostridial toxin substrate in which the lanthanide donor complex includes carbostyryl124 as the antenna. In another embodiment, a method of the invention is practiced with a clostridial toxin substrate in which the lanthanide donor complex is CS124-DTPA-EMCH-Tb.

[082] A method of the invention can be practiced with a clostridial toxin substrate which incorporates any of a variety of acceptors including, without limitation, acceptor fluorophores such as green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP). In one embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes green fluorescent protein as the acceptor. Non-fluorescent acceptors such as heme proteins also are useful in the methods of the invention.

[083] It is understood that a method of the invention can be practiced using a clostridial toxin substrate which includes any of a variety of recognition sequences. In one embodiment, the recognition sequence is a BoNT/A recognition sequence such as, without limitation, a BoNT/A recognition sequence containing at least six consecutive residues of SNAP-25, where the six consecutive residues include Gln-Arg, or a peptidomimetic thereof. Such a BoNT/A recognition sequence can include, for example, residues 134 to 206 of SEQ ID NO: 2. A recognition sequence useful in a method of the invention also can be, without limitation, a BoNT/B recognition sequence. Such a BoNT/B recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof. In a further embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a BoNT/C1 recognition sequence. Such a BoNT/C1 recognition sequence can contain, without limitation, at least six consecutive residues of syntaxin, where the six consecutive residues include Lys-Ala, or a peptidomimetic thereof. A BoNT/C1 recognition sequence useful in the invention also can contain at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ala, or a peptidomimetic thereof.

[084] In a further embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a BoNT/D recognition sequence. Such a BoNT/D recognition sequence can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Lys-Leu, or a peptidomimetic thereof. A recognition sequence useful in the invention also can be, for example, a BoNT/E recognition sequence. Such a BoNT/E recognition sequence can contain, without limitation, at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ile, or a peptidomimetic thereof. In yet another embodiment, a method of the invention is practiced with a clostridial toxin substrate which includes a BoNT/F recognition sequence. BoNT/F recognition sequences useful in the invention encompass, without limitation, those having at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Lys, or a peptidomimetic thereof. A method of the invention additionally can be practiced with a clostridial toxin substrate which includes a BoNT/G recognition sequence. Such BoNT/G recognition sequences encompass, without limitation, those having at least six consecutive residues of VAMP, where the six consecutive residues include Ala-Ala, or a peptidomimetic thereof. In still a further embodiment, a recognition sequence useful in the invention is a TeNT recognition sequence. Such a TeNT recognition sequence can be, without limitation, a sequence containing at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof.

[085] In particular embodiments, a method of the invention is practiced with a clostridial toxin substrate, such as one including a lanthanide donor complex in which the lanthanide ion is a terbium ion or one in which the lanthanide-binding site includes the coordination site of an EF hand motif, which is a peptide or peptidomimetic having at most 300 residues. In a further embodiment, a method of the invention is practiced with a clostridial toxin substrate which is a peptide or peptidomimetic having at most 150 residues. In a method of the invention, a clostridial toxin substrate of the invention can be cleaved with a...

range of activities. In one embodiment, a method of the invention is practiced under conditions such that the clostridial toxin substrate is cleaved with an activity of at least 1 nanomole/minute/milligram toxin. In another embodiment, a method of the invention is practiced under conditions such that the clostridial toxin substrate is cleaved with an activity of at least 20 nanomoles/minute/milligram toxin. In a further embodiment, a method of the invention is practiced under conditions such that the clostridial toxin substrate is cleaved with an activity of at least 100 nanomoles/minute/milligram toxin. The methods of the invention can be useful for determining the presence or activity of a clostridial toxin in any of a variety of samples including, but not limited to, crude cell lysates; isolated clostridial toxins such as isolated clostridial toxin light chains; and formulated clostridial toxin products including, but not limited to, formulated BoNT/A, BoNT/B and BoNT/E toxin products.

[086] As discussed further below, it is understood that the methods of the invention are applicable to crude samples as well as highly purified dichain and single chain toxins. As non-limiting examples, a method of the invention can be useful to determine the presence or activity of a clostridial toxin in a food or beverage sample; to assay a sample from a human or animal, for example, exposed to a clostridial toxin or having one or more symptoms of a clostridial toxin; to follow activity during production and purification of clostridial toxin; or to assay formulated clostridial toxin products such as pharmaceuticals or cosmetics.

[087] A variety of samples are useful in the methods of the invention. As used herein, the term "sample" means any biological matter that contains or potentially contains an active clostridial toxin. Thus, the term sample encompasses, but is not limited to, purified or partially purified clostridial toxin; recombinant single chain or dichain toxin with a naturally or non-naturally occurring sequence; recombinant clostridial toxin with a modified protease specificity; recombinant clostridial toxin with an altered cell specificity; chimeric toxin containing structural elements from multiple clostridial toxin species or subtypes; bulk toxin; formulated toxin product; cells or crude, fractionated or partially purified cell lysates, for example, engineered to include a recombinant nucleic acid encoding a clostridial toxin; bacterial, baculoviral and yeast lysates; raw, cooked, partially cooked or processed foods; beverages; animal feed; soil samples; water samples; pond sediments; lotions; cosmetics; and clinical formulations. It further 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.

[088] As discussed further below, a variety of conditions suitable for clostridial toxin protease activity are useful in the methods of the invention. For example, conditions suitable for clostridial toxin protease activity can be provided such that at least 10% of the substrate is cleaved. Similarly, conditions suitable for clostridial toxin protease activity can be provided such that at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the clostridial toxin substrate is cleaved, or such that 100% of the clostridial toxin

substrate is cleaved. In one embodiment, the conditions suitable for clostridial toxin protease activity are provided such that the assay is linear. In another embodiment, conditions suitable for clostridial toxin protease activity are provided such that at least 90% of the clostridial toxin substrate is cleaved. In a further embodiment, conditions suitable for clostridial toxin protease activity are provided such that at most 25% of the clostridial toxin substrate is cleaved. In yet further embodiments, conditions suitable for clostridial toxin protease activity are provided such that at most 5%, 10%, 15% or 20% of the clostridial toxin substrate is cleaved.

[089] In the methods of the invention, the clostridial toxin substrate can be treated with a sample in solution phase. As used herein in reference to a clostridial toxin substrate, the term "in solution phase" means that the substrate is soluble and, during proteolysis, is not constrained or immobilized on a solid support such as a bead, column or dish.

[090] In the methods of the invention, a sample is treated with a clostridial toxin substrate under conditions suitable for clostridial toxin protease activity. Exemplary conditions suitable for clostridial toxin protease activity are well known in the art, and further can be determined by routine methods. See, for example, Hallis et al., *J. Clin. Microbiol.* 34:1934-1938 (1996); Ekong et al., *Microbiol.* 143:3337-3347 (1997); Shone et al., WO 95/33850; Schmidt and Bostian, *supra*, 1995; Schmidt and Bostian, *supra*, 1997; Schmidt et al., *supra*, 1998; and Schmidt and Bostian, U.S. Patent No. 5,965,699. It is understood that conditions suitable for clostridial toxin protease activity can depend, in part, on the specific clostridial toxin type or subtype being assayed and the purity of the toxin preparation. Conditions suitable for clostridial toxin protease activity generally include a buffer, such as HEPES, Tris or sodium phosphate, typically in the range of pH 5.5 to 9.5, for example, in the range of pH 6.0 to 9.0, pH 6.5 to 8.5 or pH 7.0 to 8.0. Conditions suitable for clostridial toxin protease activity also can include, if desired, dithiothreitol, β -mercaptoethanol or another reducing agent, for example, where a dichain toxin is being assayed (Ekong et al., *supra*, 1997). In one embodiment, the conditions include DTT in the range of 0.01 mM to 50 mM; in other embodiments, the conditions include DTT in the range of 0.1 mM to 20 mM, 1 to 20 mM, or 5 to 10 mM. If desired, an isolated clostridial toxin or sample can be pre-incubated with a reducing agent, for example, with 10 mM dithiothreitol (DTT) for about 30 minutes prior to addition of clostridial toxin substrate.

[091] Clostridial toxins are zinc metalloproteases, and a source of zinc, such as zinc chloride or zinc acetate, typically in the range of 1 to 500 μ M, for example, 5 to 10 μ M can be included, if desired, as part of the conditions suitable for clostridial toxin protease activity. One skilled in the art understands that zinc chelators such as EDTA generally are excluded from a buffer for determining the presence or activity of a clostridial toxin.

[092] Conditions suitable for clostridial toxin protease activity can optionally include a detergent such as TWEEN-20, which can be used, for example, in place of bovine serum albumin. TWEEN-20 can be

provided, for example, in the range of 0.001% to 10% (v/v), or in the range of 0.01% to 1.0% (v/v). As a non-limiting example, TWEEN-20 can be included at a concentration of 0.1 % (v/v).

[093] Conditions suitable for clostridial toxin protease activity also can include, if desired, bovine serum albumin (BSA) or another agent which acts as a protein stabilizer, solubilizing agent or blocker of surface loss. As an example, when included, BSA typically is provided in the range of 0.1 mg/ml to 10 mg/ml. In one embodiment, BSA is included at a concentration of 1 mg/ml. See, for example, Schmidt and Bostian, *supra*, 1997. In another embodiment, BSA is included at a concentration of 0.1% (w/v).

[094] The amount of clostridial toxin substrate can be varied in a method of the invention. A clostridial toxin substrate can be supplied, for example, at a concentration of 1 μ M to 500 μ M, 1 μ M to 50 μ M, 1 μ M to 30 μ M, 5 μ M to 20 μ M, 50 μ M to 3.0 mM, 0.5 mM to 3.0 mM, 0.5 mM to 2.0 mM, or 0.5 mM to 1.0 mM. The skilled artisan understands that the concentration of clostridial toxin substrate or the amount of sample can be limited, if desired, such that the assay is linear. In one embodiment, a method of the invention relies on a clostridial toxin substrate concentration of less than 100 μ M. In further embodiments, a method of the invention relies on a clostridial toxin substrate concentration of less than 50 μ M or less than 25 μ M. In a further embodiment, a method of the invention relies on a clostridial toxin substrate concentration of 10 μ M to 20 μ M. If desired, a linear assay also can be performed by mixing clostridial toxin substrate with corresponding, "unlabeled" substrate which lacks a functional lanthanide donor complex. The appropriate dilution can be determined, for example, by preparing serial dilutions of clostridial toxin substrate in the corresponding unlabeled substrate.

[095] The concentration of purified or partially purified clostridial toxin to be assayed in a method of the invention generally is in the range of about 0.0001 ng/ml to 500 μ g/ml toxin, for example, about 0.0001 ng/ml to 50 μ g/ml toxin, 0.001 ng/ml to 500 μ g/ml toxin, 0.001 ng/ml to 50 μ g/ml toxin, 0.0001 to 5000 ng/ml toxin, 0.001 ng/ml to 5000 ng/ml, 0.01 ng/ml to 5000 ng/ml, 0.1 ng/ml to 5000 ng/ml, 1 ng/ml to 5000 ng/ml, 10 ng/ml to 5000 ng/ml, 50 ng/ml to 5000 ng/ml, 50 ng/ml to 500 ng/ml or 100 ng/ml to 5000 ng/ml toxin, which can be, for example, purified recombinant dichain or single chain toxin or formulated clostridial toxin product containing human serum albumin and excipients. Generally, the amount of purified toxin assayed in a method of the invention is in the range of 0.1 pg to 100 μ g, for example, 0.1 pg to 50 μ g or 0.1 pg to 10 μ g.

[096] The concentration of purified or partially purified clostridial toxin assayed in a method of the invention can be, for example, in the range of about 0.1 pM to 100 μ M, 0.1 pM to 10 μ M, 0.1 pM to 1 μ M, 0.1 pM to 500 nM, 0.1 pM to 100 nM, for example, 1 pM to 2000 pM, 1 pM to 200 pM, 1 pM to 50 pM, 1 nM to 1 μ M, 1 nM to 500 nM, 1 nM to 200 nM, 1 nM to 100 nM, or 3 nM to 100 nM toxin, which can be, for example, purified native or recombinant light chain or dichain toxin or formulated clostridial toxin product containing human serum albumin and excipients. In particular embodiments, the concentration of purified or partially purified recombinant BoNT/A, BoNT/B or BoNT/E light chain or dichain or formulated toxin

product is in the range of 1 pM to 2000 pM, 10 pM to 2000 pM, 20 pM to 2000 pM, 40 pM to 2000 pM, or 1 pM to 200 pM. In further embodiments, the concentration of purified or partially purified recombinant BoNT/C light chain or dichain or formulated toxin product is in the range of 1 nM to 200 nM, 4 nM to 100 nM, 10 nM to 100 nM or 4 nM to 60 nM. One skilled in the art understands that the concentration of purified or partially purified clostridial toxin will depend on the serotype of the toxin assayed, as well as the purity or recombinant sequence of the toxin, the presence of inhibitory components, and the assay conditions. It is additionally understood that purified, partially purified or crude samples can be diluted to within a convenient range for assaying for clostridial toxin protease activity against a standard curve. Similarly, it is understood that a sample can be diluted, if desired, such that the assay is linear.

[097] Conditions suitable for clostridial toxin protease activity also generally include, for example, temperatures in the range of about 20°C to about 45°C, for example, in the range of 25°C to 40°C, or the range of 35°C to 39°C. Assay volumes often are in the range of about 5 µl to about 200 µl, for example, in the range of about 10 µl to 100 µl or about 0.5 µl to 100 µl, although nanoliter reaction volumes also can be used with the methods of the invention. Assay volumes also can be, for example, in the range of 100 µl to 2.0 ml or in the range of 0.5 ml to 1.0 ml.

[098] Assay times can be varied as appropriate by the skilled artisan and generally depend, in part, on the concentration, purity and activity of the clostridial toxin. Assay times generally vary, without limitation, in the range of about 15 minutes to about 5 hours. As non-limiting examples, exemplary assay times include incubation, for example, at 37°C for 30 minutes, 45 minutes, 60 minutes, 75 minutes or 90 minutes. In particular embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of the clostridial toxin substrate is cleaved. In further embodiments, the protease reaction is stopped before more than 5%, 10%, 15%, 20%, 25% or 50% of the clostridial toxin substrate is cleaved. It is understood that protease reactions can be terminated by the appropriate reagent, which generally depends on the lanthanide donor complex and other components of the substrate. As a non-limiting example, a protease reaction based on a substrate containing GFP as the fluorescent acceptor can be terminated by the addition of guanidinium chloride, for example, to a final concentration of 1 to 2 M. Protease reactions also can be terminated by addition of H₂SO₄; addition of about 0.5 to 1.0 sodium borate, pH 9.0 to 9.5; or addition of zinc chelators. One skilled in the art understands that protease reactions can be terminated, if desired, prior to exciting the antenna.

[099] As a non-limiting example, conditions suitable for clostridial toxin protease activity such as BoNT/A protease activity can be incubation at 37°C for 90 minutes in a buffer containing 50 mM HEPES (pH 7.2), 10 µM ZnCl₂, 10 mM DTT, and 0.1% (v/v) TWEEN-20 with 10-16 µM substrate. If desired, samples containing BoNT/A, particularly dichain BoNT/A, can be preincubated with dithiothreitol, for example, for 20 or 30 minutes before addition of substrate. As a further non-limiting example, conditions suitable for BoNT/A protease activity can be incubation at 37°C in a buffer such as 30 mM HEPES (pH 7.3) containing a reducing agent such as 5 mM dithiothreitol; and a source of zinc such as 25 µM zinc

chloride (approximately 7 nM; Schmidt and Bostian, *supra*, 1997). BSA in the range of 0.1 mg/ml to 10 mg/ml, for example, 1 mg/ml BSA, also can be included when a sample is treated with a clostridial toxin substrate (Schmidt and Bostian, *supra*, 1997). As another non-limiting example, conditions suitable for clostridial toxin protease activity, for example BoNT/B activity, can be incubation in 50 mM HEPES, pH 7.4, with 10 μ M zinc chloride, 1% fetal bovine serum and 10 mM dithiothreitol, with incubation for 90 minutes at 37°C (Shone and Roberts, *Eur. J. Biochem.* 225:263-270 (1994); Hallis et al., *supra*, 1996); or can be, for example, incubation in 40 mM sodium phosphate, pH 7.4, with 10 mM dithiothreitol, optionally including 0.2% (v/v) Triton X-100, with incubation for 2 hours at 37°C (Shone et al., *supra*, 1993). Conditions suitable for tetanus toxin protease activity or other clostridial toxin protease activity can be, for example, incubation in 20 mM HEPES, pH 7.2, and 100 mM NaCl for 2 hours at 37°C with 25 μ M peptide substrate (Cornille et al., *supra*, 1994).

[0100] The present invention relies, in part, on luminescence resonance energy transfer (LRET), in which a lanthanide ion such as Tb³⁺ or Eu³⁺ transfers energy non-radiatively to an organic acceptor, which may be a fluorophore, through intramolecular long-range dipole-dipole coupling. FRET is dependent on the inverse sixth power of the intramolecular separation of the lanthanide donor complex and acceptor, and for effective transfer, the lanthanide donor complex and acceptor are in close proximity, separated, for example, by about 10 Å to about 100 Å. Effective energy transfer is dependent on the spectral characteristics of the lanthanide donor complex and acceptor as well as their relative orientation (see Clegg, *Current Opinion in Biotech.* 6:103-110 (1995); and Selvin, *Nature Structural Biol.* 7:730-734 (2000)).

[0101] In a clostridial toxin substrate of the invention, a lanthanide ion and acceptor are selected so that the lanthanide donor complex and acceptor exhibit resonance energy transfer when the antenna of the lanthanide donor complex is excited. As is well known in the art, the efficiency of resonance energy transfer is dependent on the separation distance of the lanthanide ion or other component of the lanthanide donor complex and acceptor as described by the Förster equation, as well as the fluorescent quantum yield of the lanthanide ion and the energetic overlap with the acceptor. In one embodiment, the invention provides a clostridial toxin substrate in which, under optimal conditions, the efficiency of LRET between the lanthanide donor complex and acceptor is at least 10%. In another embodiment, the invention provides a clostridial toxin substrate in which, under optimal conditions, the efficiency of LRET between the lanthanide donor complex and acceptor is at least 20%. In still further embodiments, the invention provides a clostridial toxin substrate in which, under optimal conditions, the efficiency of LRET between the lanthanide donor complex and acceptor is at least 30%, 40%, 50%, 60%, 70% or 80%.

[0102] The clostridial toxin substrates of the invention exploit the remarkable luminescent properties of lanthanides, which are their long, millisecond to submillisecond lifetimes, narrow and multiple emission bands in the visible spectrum, and unpolarized emission. Useful lanthanide donor complex/acceptor pairs for use in the clostridial toxin substrates of the invention include, without limitation, CS124-DTPA-EMCH-

Tb or another terbium ion complex in combination with a green fluorescent protein or blue fluorescent protein as the acceptor (see Examples I and II). A useful lanthanide donor complex/acceptor pair also can be the lanthanide donor complex Eu-trisbipyridine cryptate (TBP-Eu³⁺, λ_{Ex} 337 nm) in combination with the 105 kDa phycobiliprotein acceptor fluorophore, allophycocyanin (Sittampalam et al., Curr. Opin. Chem. Biol. 1:384-391 (1997)). The Eu-trisbipyridine cryptate has two bipyridyl groups that harvest light and channel it to the caged Eu³⁺; Eu³⁺ nonradiatively transfers energy to allophycocyanin when in close proximity to the acceptor, exhibiting greater than 50% transfer efficiency at a lanthanide ion-acceptor distance of 9.5 nm. Furthermore, both TBP-Eu³⁺ and allophycocyanin and their spectroscopic characteristics are very stable in biological media, and allophycocyanin emits (λ_{Em} 665 nm) with the long lifetime of the lanthanide ion, allowing time-resolved detection (Kolb et al., J. Biomol. Screening 1:203-210 (1996)). Methods of preparing substrates containing such donor fluorophore-acceptor pairs are well known in the art as described, for example, in Kolb et al., *supra*, 1996, and Sittampalam et al., *supra*, 1997.

[0103] A clostridial toxin substrate of the invention contains a clostridial toxin cleavage site which is positioned between a lanthanide donor complex and an acceptor. In one embodiment, the lanthanide donor complex is positioned carboxy-terminal of the cleavage site while the acceptor is positioned amino-terminal of the cleavage site. In another embodiment, the lanthanide donor complex is positioned amino-terminal of the cleavage site while the acceptor is positioned carboxy-terminal of the cleavage site.

[0104] One skilled in the art understands that there are several considerations in selecting and positioning a lanthanide donor complex and acceptor in a clostridial toxin substrate of the invention. The lanthanide donor complex and acceptor generally are positioned to minimize interference with substrate binding to, or proteolysis by, the clostridial toxin. Thus, a lanthanide donor complex and acceptor can be selected and positioned, for example, so as to minimize the disruption of bonded and non-bonded interactions that are important for binding, and to minimize steric hindrance. In addition, the spatial distance between the acceptor and lanthanide donor complex generally is limited to achieve efficient energy transfer from the lanthanide donor complex to the acceptor.

[0105] As discussed above, efficiency of energy transfer from lanthanide donor complex to acceptor will be dependent, in part, on the spatial separation of the lanthanide donor complex and acceptor. As the distance between the lanthanide donor complex and acceptor increases, there is less energy transfer to the acceptor, and the lanthanide donor complex signal therefore increases, even prior to cleavage. The overall increase in fluorescence yield of the lanthanide donor complex, upon cleavage of the substrate, is dependent upon many factors, including the separation distance between the lanthanide donor complex and acceptor in the substrate, the spectral overlap between the lanthanide donor complex and acceptor, and the concentration of substrate used in an assay. One skilled in the art understands that, as the concentration of substrate increases, intermolecular quenching of the donor, even after proteolytic cleavage, can become a factor. This phenomenon is denoted the "inner filter effect."

[0106] The Förster distance, which is the separation between a donor fluorophore and an acceptor for 50% energy transfer, represents a spatial separation between donor fluorophore and acceptor that provides a good sensitivity. For peptide substrates, adjacent residues are separated by a distance of approximately 3.6 Å in the most extended conformation. However, because peptides and peptidomimetics in solution rarely have a fully extended conformation, a lanthanide donor complex and an acceptor can be more widely separated than expected based on a calculation performed using 3.6 Å per residue and still remain within the Förster distance. In one embodiment, the invention provides a clostridial toxin substrate in which the lanthanide ion or other component of a lanthanide donor complex is spatially separated from an acceptor by a distance of at most 100Å. In other embodiments, the invention provides a clostridial toxin substrate in which the lanthanide ion or other component of a lanthanide donor complex is spatially separated from an acceptor by a distance of at most 90Å, 80Å, 70Å, 60Å, 50Å, 40Å, 30Å or 20Å. In further embodiments, the invention provides a clostridial toxin substrate in which the lanthanide ion or other component of a lanthanide donor complex is spatially separated from an acceptor by a distance of 10Å to 100Å, 10Å to 80Å, 10Å to 60Å, 10Å to 40Å, 10Å to 20Å, 20Å to 100Å, 20Å to 80Å, 20Å to 60Å, 20Å to 40Å, 40Å to 100Å, 40Å to 80Å or 40Å to 60Å.

[0107] One skilled in the art understands that a clostridial toxin substrate of the invention can be designed, in part, to optimize the efficiency of resonance energy transfer. One skilled in the art understands that lanthanide ions useful in the invention generally have a high quantum yield, and that an acceptor can be selected, if desired, with a high extinction coefficient to maximize the Förster distance. One skilled in the art further understands that fluorescence arising from direct excitation of an acceptor can be difficult to distinguish from fluorescence resulting from resonance energy transfer. Thus, it is recognized that a lanthanide donor complex and acceptor can be selected which have relatively little overlap of their excitation spectra such that the antenna of a lanthanide donor complex can be excited at a wavelength that does not result in direct excitation of the acceptor. It further is recognized that a clostridial toxin substrate of the invention can be designed so that the emission spectra of the lanthanide donor complex and acceptor overlap relatively little such that the two emissions can be readily distinguished. If desired, an acceptor having a high fluorescence quantum yield can be selected.

[0108] Proteolysis of a clostridial toxin substrate, and hence the presence or activity of a clostridial toxin, can be detected by a variety of means, for example, by detecting increased luminescence from at least one emission peak of a lanthanide donor complex; by detecting decreased acceptor fluorescence intensity; or by detecting a decreased ratio of fluorescence amplitudes near the acceptor emission maximum to the fluorescence amplitudes near the lanthanide donor complex emission maximum. It is understood that the relevant luminescence intensities are detected at the appropriate selected wavelength or range of wavelengths. Proteolysis of a clostridial toxin substrate, and hence the presence or activity of a clostridial toxin, also can be detected by, for example, a shift in emission maxima from near the acceptor emission maximum to near an emission maximum of the lanthanide ion, or an increased excited state lifetime of the lanthanide ion.

[0109] In one embodiment, luminescence intensity of at least one emission peak of the lanthanide donor complex is detected, with increased luminescence intensity indicative of the presence or activity of clostridial toxin. Such increased intensity can be, for example, at least two-fold, three-fold, five-fold, ten-fold, twenty-fold or more relative to luminescence intensity at the same wavelength of the same clostridial toxin substrate not contacted with sample. Such increased intensity also can be, for example, an increase of at least 0.1-fold, 0.2-fold, 0.3-fold, 0.4-fold, 0.5-fold, 0.6-fold, 0.7-fold, 0.8-fold, 0.9-fold, 1.0-fold, or 1.5-fold relative to luminescence intensity at the same wavelength of the same clostridial toxin substrate not contacted with sample.

[0110] For detection of luminescence intensity of a lanthanide donor complex, excitation is set at the wavelength of antenna absorption, and the emission of at least one peak of the lanthanide donor complex is monitored. The emission wavelength of the lanthanide donor complex generally is selected such that little or no contribution from acceptor fluorescence is observed. The presence of acceptor quenches luminescence from the lanthanide donor complex as disclosed herein in Example II. The methods of the invention for determining the presence or activity of a clostridial toxin involve determining resonance energy transfer of a clostridial toxin substrate treated with a sample relative to a control substrate and can be practiced as "fixed-time" assays or as continuous time assays.

[0111] In the methods of the invention, luminescence resonance energy transfer of the clostridial toxin treated substrate is determined relative to a control substrate. Such a control substrate can be, without limitation, the same clostridial toxin substrate which is not treated with any sample, or which is treated with a defined sample known to contain one or more clostridial toxins, or with a defined sample known to lack active clostridial toxin. It is clear from the above that a variety of control substrates are useful in the methods of the invention and that a control substrate can be a positive control substrate or a negative control substrate. A control substrate can be, for example, a negative control substrate such as a similar or identical substrate that is contacted with a similar sample that does not contain active clostridial toxin, or that is not contacted with any sample, or which is not susceptible to cleavage by the clostridial toxin. A control substrate also can be, for example, a positive control substrate such as a cleavage product that results from clostridial toxin proteolysis of the clostridial toxin substrate. Such a control substrate can be the lanthanide donor complex-containing cleavage product, the acceptor-containing cleavage product, or a combination of both.

[0112] It is understood that the methods of the invention can be automated and can be configured in a high-throughput or ultra high-throughput format using, without limitation, 96-well, 384-well or 1536-well plates. As one example, fluorescence emission can be detected using Molecular Devices FLIPR[®] instrumentation system (Molecular Devices; Sunnyvale, CA), which is designed for 96-well plate assays (Schroeder et al., *J. Biomol. Screening* 1:75-80 (1996)). FLIPR utilizes a water-cooled 488 nm argon ion laser (5 watt) or a xenon arc lamp and a semiconfocal optical system with a charge-coupled device (CCD).

camera to illuminate and image the entire plate. The FPM-2 96-well plate reader (Folley Consulting and Research; Round Lake, Illinois) also can be useful in determining resonance energy transfer in the methods of the invention. One skilled in the art understands that these and other automated systems with the appropriate spectroscopic compatibility such as the ECLIPSE cuvette reader (Varian-Cary; Walnut Creek, CA), the SPECTRA_{max} GEMINI XS (Molecular Devices) and other systems from, for example, Perkin Elmer can be useful in the methods of the invention.

[0113] Many compounds and proteins present in biological samples are naturally fluorescent; thus, the use of conventional fluorophores can lead to significant limitations in sensitivity. However, non-specific background fluorescence is short-lived, typically having a decay time of only about 10 nanoseconds and therefore dying away much earlier than sample fluorescence. Thus, most background signals can be readily differentiated using time-resolved fluorescence (TRF), which is a quick and convenient assay based on the long-lived fluorescence of the rare earth lanthanides. In time-resolved fluorescence, the detector is gated for a short period of time such that the initial burst of fluorescence, including most of the background fluorescence, is not measured. After the gating period, the longer lasting fluorescence in the sample is measured, substantially enhancing sensitivity. As a non-limiting example, a pulsed excitation source for exciting the antenna of a lanthanide donor complex can be generated using a nitrogen laser (337 nm). Typically, a pulse-width of about 5 nanoseconds is utilized with a 20 to 50-Hz repetition rate. For lifetime measurements, a photomultiplier tube with suitable color filters and counting electronics can be used. For time-delayed spectra, a spectrometer, generally utilizing diffraction gratings, and either a time-gated photomultiplier tube or a CCD, gated electronically or with a mechanical chopper are used. Such instruments are commercially available and are well known in the art as described, for example, in Xiao and Selvin, Rev. Sci. Inst., 70:3877-3881 (1999), Xiao and Selvin, J. Am. Chem. Soc. 123:7067-7073 (2001), and Selvin, *supra*, 2002.

[0114] Specific and distinct cleavage sites for different clostridial toxins are well known in the art. BoNT/A cleaves a Gln-Arg bond; BoNT/B and TeNT cleave a Gln-Phe bond; BoNT/C1 cleaves a Lys-Ala or Arg-Ala bond; BoNT/D cleaves a Lys-Leu bond; BoNT/E cleaves an Arg-Ile bond; BoNT/F cleaves a Gln-Lys bond; and BoNT/G cleaves an Ala-Ala bond (see Table A). In standard nomenclature, the sequence surrounding a clostridial toxin cleavage site is denoted P₅-P₄-P₃-P₂-P₁-P₁'-P₂'-P₃'-P₄'-P₅', with P₁-P₁' representing the scissile bond. It is understood that a P₁ or P₁' site, or both, can be substituted with another amino acid or amino acid mimetic in place of the naturally occurring residue. As an example, BoNT/A substrates have been prepared in which the P₁ position (Gln) is modified to be an alanine, 2-aminobutyric acid or asparagine residue; these substrates were hydrolyzed by BoNT/A at the P₁-Arg bond (Schmidt and Bostian, J. Protein Chem. 16:19-26 (1997)). While it is recognized that substitutions can be introduced at the P₁ position of the scissile bond, for example, a BoNT/A scissile bond, it is further recognized that conservation of the P₁' residue can be advantageous (Vaidyanathan et al., J. Neurochem. 72:327-337 (1999)). Thus, in particular embodiments, the invention provides a method

which relies on a clostridial toxin substrate having a clostridial toxin recognition sequence in which the P₁' residue is not modified or substituted relative to the naturally occurring residue in a target protein cleaved by the clostridial toxin. In other embodiments, the invention provides a method which relies on a clostridial toxin substrate having a recognition sequence in which the P₁ residue is modified or substituted relative to the naturally occurring residue in a target protein cleaved by the clostridial toxin; such a clostridial toxin substrate retains susceptibility to peptide bond cleavage between the P₁ and P₁' residues.

TABLE A BONDS CLEAVED IN HUMAN VAMP-2, SNAP-25 OR SYNTAXIN			
Toxin	Target	P₄-P₃-P₂-P₁ -- P₁'-P₂'-P₃'-P₄'	SEQ ID NO:
BoNT/A	SNAP-25	Glu-Ala-Asn- Gln—Arg* -Ala-Thr-Lys	22
BoNT/B	VAMP-2	Gly-Ala-Ser- Gln—Phe* -Glu-Thr-Ser	23
BoNT/C1	syntaxin	Asp-Thr-Lys- Lys—Ala* -Val-Lys-Tyr	24
BoNT/D	VAMP-2	Arg-Asp-Gln- Lys—Leu* -Ser-Glu-Leu	25
BoNT/E	SNAP-25	Gln-Ile-Asp- Arg—Ile* -Met-Glu-Lys	26
BoNT/F	VAMP-2	Glu-Arg-Asp- Gln—Lys* -Leu-Ser-Glu	27
BoNT/G	VAMP-2	Glu-Thr-Ser- Ala—Ala* -Lys-Leu-Lys	28
TeNT	VAMP-2	Gly-Ala-Ser- Gln—Phe* -Glu-Thr-Ser	29

* Scissile bond shown in bold

[0115] SNAP-25, VAMP and syntaxin share a short motif located within regions predicted to adopt an α -helical conformation. This motif is present in SNAP-25, VAMP and syntaxin isoforms expressed in animals sensitive to the neurotoxins. In contrast, *Drosophila* and yeast homologs that are resistant to these neurotoxins and syntaxin isoforms not involved in exocytosis contain sequence variations in the α -helical motif regions of these VAMP and syntaxin proteins.

[0116] Multiple repetitions of the α -helical motif are present in proteins sensitive to cleavage by clostridial toxins: Four copies are naturally present in SNAP-25; two copies are naturally present in VAMP; and two copies are naturally present in syntaxin. Furthermore, peptides corresponding to the specific sequence of the α -helical motifs can inhibit neurotoxin activity *in vitro* and *in vivo*, and such peptides can cross-inhibit different neurotoxins. In addition, antibodies raised against such peptides can cross-react among the three target proteins, indicating that this α -helical motif is exposed on the protein surface and adopts a similar configuration in each of the three target proteins. Consistent with these findings, SNAP-25-specific, VAMP-specific and syntaxin-specific neurotoxins cross-inhibit each other by competing for the same binding site, although they do not cleave targets non-specifically. These results indicate that a clostridial toxin recognition sequence can include, if desired, at least one α -helical motif. It is recognized

that an α -helical motif is not required for cleavage by a clostridial toxin, as evidenced by 16-mer and 17-mer substrates for BoNT/A known in the art.

[0117] Although multiple α -helical motifs are found in the naturally occurring SNAP-25, VAMP and syntaxin target proteins, a clostridial toxin recognition sequence useful in a clostridial toxin substrate can have a single α -helical motif. In particular embodiments, a method of the invention relies on a clostridial toxin recognition sequence including two or more α -helical motifs. A BoNT/A or BoNT/E recognition sequence can include, for example, the S4 α -helical motif, alone or combined with one or more additional α -helical motifs; a BoNT/B, BoNT/G or TeNT recognition sequence can include, for example, the V2 α -helical motif, alone or combined with one or more additional α -helical motifs; a BoNT/C1 recognition sequence can include, for example, the S4 α -helical motif, alone or combined with one or more additional α -helical motifs, or the X2 α -helical motif, alone or combined with one or more additional α -helical motifs; and a BoNT/D or BoNT/F recognition sequence can include, for example, the V1 α -helical motif, alone or combined with one or more additional α -helical motifs.

[0118] BoNT/A recognition sequences

[0119] As used herein, the term "botulinum toxin serotype A recognition sequence" is synonymous with "BoNT/A recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/A under conditions suitable for clostridial toxin protease activity. A scissile bond cleaved by BoNT/A can be, for example, Gln-Arg.

[0120] A variety of BoNT/A recognition sequences are well known in the art and are useful in the invention. A BoNT/A recognition sequence can have, for example, residues 134 to 206 or residues 137 to 206 of human SNAP-25 (Ekong et al., *supra*, 1997; U.S. Patent No. 5,962,637). A BoNT/A recognition sequence also can include, without limitation, the sequence Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 30) or a peptidomimetic thereof, which corresponds to residues 190 to 202 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 31) or a peptidomimetic thereof, which corresponds to residues 187 to 201 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 32) or a peptidomimetic thereof, which corresponds to residues 187 to 202 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu (SEQ ID NO: 33) or a peptidomimetic thereof, which corresponds to residues 187 to 203 of human SNAP-25; Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 34) or a peptidomimetic thereof, which corresponds to residues 186 to 202 of human SNAP-25; or Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu (SEQ ID NO: 35) or a peptidomimetic thereof, which corresponds to residues 186 to 203 of human SNAP-25. See, for example, Schmidt and Bostian, *J. Protein Chem.* 14:703-708 (1995); Schmidt and Bostian, *supra*, 1997; Schmidt et

al., FEBS Letters 435:61-64 (1998); and Schmidt and Bostian, U.S. Patent No. 5,965,699). If desired, a similar BoNT/A recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/A-sensitive SNAP-25 isoform or homolog such as, for example, murine, rat, goldfish or zebrafish SNAP-25 or can be any of the peptides described herein or known in the art, for example, in U.S. Patent No. 5,965,699.

[0121] A BoNT/A recognition sequence useful in the invention can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype A, or can be substantially similar to a segment of a BoNT/A-sensitive protein. As illustrated in Table B, a variety of naturally occurring proteins sensitive to cleavage by BoNT/A are known in the art and include, for example, human, mouse and rat SNAP-25; and goldfish SNAP-25A and SNAP-25B. Thus, a BoNT/A recognition sequence useful in the invention can correspond, for example, to a segment of human SNAP-25, mouse SNAP-25, rat SNAP-25, goldfish SNAP-25A or 25B, or another naturally occurring protein sensitive to cleavage by BoNT/A. Furthermore, comparison of native SNAP-25 amino acid sequences cleaved by BoNT/A reveals that such sequences are not absolutely conserved (see Table B and Figure 3), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/A-sensitive SNAP-25 sequence can be tolerated in a BoNT/A recognition sequence useful in the invention.

TABLE B

TABLE B
Cleavage of SNAP-25 and related proteins^{a,b,c,d}

Species	Isoform		Cleavage Sites			SEQ ID NO:	Resistance to Cleavage by
			BoNT/E		BoNT/A	BoNT/C	
human		174					
mouse	SNAP-25		qnrqid	ri	mekadsnktridean	qra	tkmlgsg
rat							206
							36
							none ^a
human	SNAP-23	180	qnrqid	ri	tdkadtnrdridian	qra	kklids
mouse	SNAP-23	179	qnrqid	ri	tekadtnknridian	qra	kklids
chicken	SNAP-25	174	qnrqid	ri	meklipikpglmkpt	qra	qrcsavvk
goldfish	SNAP-25 A	171	qnrqid	ri	mdmadsnktridean	qra	tkmlgsg
	SNAP-25 B	172	qnrqid	ri	mekadsnktridean	qra	tkmlgsg
Torpedo	SNAP-25	180	qnaqvq	ri	vllkgdmnkaridean	qra	tkml
sea urchin	SNAP-25	180	qnsqvg	ri	tskaesnegrinsad	qra	knllrnk
C-elegans	SNAP-25	203	qnrqid	ri	hdkqsnevrvesank	qra	nlitk
Drosophila	SNAP-25	182	qnrqid	ri	nrkgesneariavan	qra	hqllk
leech	SNAP-25	181	qnrqvq	ri	nnkmtnqlrisdan	qra	skllke

a = In vitro cleavage of SNAP-25 requires 1000-fold higher BoNT/C concentration than BoNT/A or /E.

b = Substitution of p182r, or k185dd (boxes) induces susceptibility toward BoNT/E.

c = Resistance to BoNT/A possibly due to d189 or e189 substitution by v189, see box.

d = Note that Torpedo is susceptible to BoNT/A.

e = Note the presence of several non-conservative mutations around putative cleavage sites.

[0122] clostridial toxin substrate, such as a substrate containing a BoNT/A recognition sequence, can have one or multiple modifications as compared to a naturally occurring sequence that is cleaved by the corresponding clostridial toxin. As an example, as compared to a 17-mer corresponding to residues 187 to 203 of human SNAP-25, substitution of sp193 with Asn in the BoNT/A substrate resulted in a relative rate of proteolysis of 0.23; substitution of Glu194 with Gln resulted in a relative rate of 2.08; substitution of Ala195 with 2-aminobutyric acid resulted in a relative rate of 0.38; and substitution of Gln197 with Asn, 2-aminobutyric acid or Ala resulted in a relative rate of 0.66, 0.25, or 0.19, respectively (see Table C). Furthermore, substitution of Ala199 with 2-aminobutyric acid resulted in a relative rate of 0.79; substitution of Thr200 with Ser or 2-aminobutyric acid resulted in a relative rate of 0.26 or 1.20, respectively; substitution of Lys201 with Ala resulted in a relative rate of 0.12; and substitution of Met202 with Ala or norleucine resulted in a relative rate of 0.38 or 1.20, respectively. See Schmidt and Bostian, *supra*, 1997. These results indicate that a variety of residues can be substituted in a clostridial toxin substrate as compared to a naturally occurring toxin-sensitive sequence. In the case of BoNT/A, these results indicate that residues including but not limited to Glu194, Ala195, Gln197, Ala199, Thr200 and Met202, Leu203, Gly204, Ser205, and Gly206, as well as residues more distal from the Gln-Arg scissile bond, can be substituted or conjugated to a fluorophore, bulking group, donor fluorophore or acceptor in a BoNT/A substrate useful in the invention. Such a BoNT/A substrate is detectably proteolyzed at the scissile bond by BoNT/A under conditions suitable for clostridial toxin protease activity. Thus, a BoNT/A substrate can include, if desired, one or several amino acid substitutions, additions or deletions relative to a naturally occurring SNAP-25 sequence.

TABLE C
KINETIC PARAMETERS OF BONT/A SYNTHETIC PEPTIDE SUBSTRATES

Peptide	Sequence ^a	SEQ ID NO:	Relative Rate ^b
[1-15]	SNKTRIDEANQRATK	31	0.03
[1-16]	SNKTRIDEANQRATKM	32	1.17
[1-17]	SNKTRIDEANQRATKML	33	1.00
M16A	SNKTRIDEANQRATK <u>A</u> L	50	0.38
M16X	SNKTRIDEANQRATK <u>X</u> L	51	1.20
K15A	SNKTRIDEANQRAT <u>A</u> ML	52	0.12
T14S	SNKTRIDEANQRAS <u>S</u> KML	53	0.26
T14B	SNKTRIDEANQRAB <u>B</u> KML	54	1.20
A13B	SNKTRIDEANQRB <u>B</u> TKML	55	0.79
Q11A	SNKTRIDEAN <u>A</u> RATKML	56	0.19
Q11B	SNKTRIDEAN <u>B</u> RATKML	57	0.25
Q11N	SNKTRIDEAN <u>N</u> RATKML	58	0.66
N10A	SNKTRIDEA <u>A</u> QRATKML	59	0.06
A9B	SNKTRIDE <u>B</u> NQRATKML	60	0.38
E8Q	SNKTRID <u>Q</u> ANQRATKML	61	2.08
D7N	SNKTRID <u>N</u> ANQRATKML	62	0.23

^a Nonstandard amino acid abbreviations are: B, 2-aminobutyric acid; X, 2-aminoheptanoic acid (norleucine)

^b Initial hydrolysis rates relative to peptide [1-17]. Peptide concentrations were 1.0 mM.

[0123] BoNT/B recognition sequences

[0124] As used herein, the term “botulinum toxin serotype B recognition sequence” is synonymous with “BoNT/B recognition sequence” and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/B under appropriate conditions. A scissile bond cleaved by BoNT/B can be, for example, Gln-Phe.

[0125] A variety of BoNT/B recognition sequences are well known in the art or can be defined by routine methods. Such BoNT/B recognition sequences can include, for example, a sequence corresponding to some or all of the hydrophilic core of a VAMP protein such as human VAMP-1 or human VAMP-2. A BoNT/B recognition sequence can include, without limitation, residues 33 to 94, residues 45 to 94, residues 55 to 94, residues 60 to 94, residues 65 to 94, residues 60 to 88 or residues 65 to 88 of human VAMP-2 (SEQ ID NO: 8), or residues 60 to 94 of human VAMP-1 (SEQ ID NO: 7). See, for example, Shone et al., Eur. J. Biochem. 217: 965-971 (1993). and U.S. Patent No. 5,962,637. If desired, a similar BoNT/B recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/B-sensitive VAMP isoform or homolog such as human VAMP-1 or rat or chicken VAMP-2.

[0126] Thus, it is understood that a BoNT/B recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype B, or can be substantially similar to such a segment of a BoNT/B-sensitive protein. As shown in Table D, a variety of naturally occurring proteins sensitive to cleavage by BoNT/B are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-2; rat cellubrevin; chicken VAMP-2; *Torpedo* VAMP-1; sea urchin VAMP; *Aplysia* VAMP; squid VAMP; *C. elegans* VAMP; *Drosophila* n-syb; and leech VAMP. Thus, a BoNT/B recognition sequence included in a BoNT/B substrate can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-2, rat cellubrevin, chicken VAMP-2, *Torpedo* VAMP-1, sea urchin VAMP, *Aplysia* VAMP, squid VAMP, *C. elegans* VAMP, *Drosophila* n-syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/B. Furthermore, as shown in Table D, comparison of native VAMP amino acid sequences cleaved by BoNT/B reveals that such sequences are not absolutely conserved (see, also, Figure 4), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring VAMP sequence can be tolerated in a BoNT/B substrate of the invention.

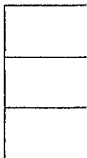
[0127] BoNT/C1 recognition sequences

[0128] As used herein, the term “botulinum toxin serotype C1 recognition sequence” is synonymous with “BoNT/C1 recognition sequence” and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/C1

under appropriate conditions. A scissile bond cleaved by BoNT/C1 can be, for example, Lys-Ala or Arg-Ala.

[0129] It is understood that a BoNT/C1 recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype C1, or can be substantially similar to a segment of a BoNT/C1-sensitive protein. As shown in Table E, a variety of naturally occurring proteins sensitive to cleavage by BoNT/C1 are known in the art and include, for example, human, rat, mouse and bovine syntaxin 1A and 1B; rat syntaxins 2 and 3; sea urchin syntaxin; *Aplysia* syntaxin 1; squid syntaxin; *Drosophila* Dsynt1; and leech syntaxin 1. Thus, a BoNT/C1 recognition sequence useful in a BoNT/C1 substrate can correspond, for example, to a segment of human, rat, mouse or bovine syntaxin 1A or 1B, rat syntaxin 2, rat syntaxin 3, sea urchin syntaxin, *Aplysia* syntaxin 1, squid syntaxin, *Drosophila* Dsynt1, leech syntaxin 1, or another naturally occurring protein sensitive to cleavage by BoNT/C1. Furthermore, comparison of native syntaxin amino acid sequences cleaved by BoNT/C1 reveals that such sequences are not absolutely conserved (see Table E and Figure 5), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/C1-sensitive syntaxin sequence can be tolerated in a BoNT/C1 substrate useful in the invention.

[0130] TABLE D

TABLE D Cleavage of VAMP ^{a,b}													
Species	Isoform	Cleavage Sites							SEQ ID NO:	Resistance to Cleavage by			
		BoNT/B TeNT											
		BoNT/F	$\begin{smallmatrix} \uparrow & \uparrow \\ \nabla & \nabla \end{smallmatrix}$	BoNT/D	$\begin{smallmatrix} \uparrow \\ \nabla \end{smallmatrix}$	$\begin{smallmatrix} \uparrow \\ \nabla \end{smallmatrix}$	BoNT/G						
human mouse bovine		VAMP-1 ⁵³	dkvlerd	qkl	selddradalqagas	qf	ess	aa	klkrkyww	⁹²	63	none	
		VAMP-2 ⁵¹	dkvlerd	qkl	selddradalqagas	qf	ets	aa	klkrkyww	⁹⁰	64	none	
rat		VAMP-1 ⁵³	dkvlerd	qkl	selddradalqagas	qf	ess	aa	klkrkyww	⁹²	65	TeNT & BoNT/B	
		VAMP-2 ⁵¹	dkvlerd	qkl	selddradalqagas	qf	ets	aa	klkrkyww	⁹⁰	66	none	
		Cellubrevin ³⁸	dkvlerd	qkl	selddradalqagas	qf	ets	aa	klkrkyww	⁷⁷	67	none	
		TI-VAMP ¹⁴⁶	dlvaqrg	qkl	elldktenlvdssv	qf	ktt	aa	nlaramcm	¹⁷⁵	68	all	
		VAMP-1 ⁻	----erd	qkl	selddradalqagas	qf	ess	aa	klkr----	-	69	TeNT & BoNT/B	
chicken		VAMP-2 ⁻	----erd	qkl	selddradalqagas	qf	ets	aa	klkr----	-	70	none	
Torpedo		VAMP-1 ⁵⁵	dkvlerd	qkl	selddradalqagas	qf	ess	aa	klkrkyww	⁹⁴	71	none	
sea urchin		VAMP ³⁵	dkvldrd	qkl	svlddradalqggas	qf	etn	aa	klkrkyww	⁷⁴	72	BoNT/F, D & G	

[0131] TABLE D

TABLE D Cleavage of VAMP ^{a,b}												
Species	Isoform	Cleavage Sites							SEQ ID NO:	Resistance to Cleavage by		
BoNT/B TeNT												
		BoNT/F	↓ ↓ ↓	BoNT/D	↓ ↓ ↓		BoNT/G					
Aplysia	VAMP ⁴¹	ekvlrd	qkl	sqlddraealqagas	qf	eas	klkrkyww	80	73	BoNT/G		
squid	VAMP ⁶⁰	dkvlrd	skl	seiddradalqagas	qf	eas	klkrkfw	99	74	BoNT/F & G		
C. elegans	VAMP ⁸⁶	nkvmrd	qkl	nsldhraevlqngas	qf	qq	tlrqkyww	115	75	BoNT/F, D & G		
	syb ^a	ekvlrd	qkl	seigeradqlcqgas	qs	eqq	klkrkqww	106	76	TeNT & BoNT/B & G		
Drosophila	n-syb ^b	ekvlrd	skl	seiddradalqagas	qf	eqq	klkrkfw	100	77	BoNT/F & G		
leech	VAMP ⁴⁹	dkvlekd	qkl	aeldgradalqagas	qf	eas	klkrkfw	88	78	BoNT/G		

a = Sequence corrected in position 93 (f>s).

b = Sequence corrected in position 68 (t>s).

[0132] TABLE E :

TABLE E
Cleavage of syntaxin

Cleavage of syntaxin									
Species	Isoform		Cleavage Sites			SEQ ID NO:	Resistance to Cleavage by		

[0133] A variety of naturally occurring SNAP-25 proteins also are sensitive to cleavage by BoNT/C1, including human, mouse and rat SNAP-25; goldfish SNAP-25A and 25B; and *Drosophila* and leech SNAP-25. Thus, a BoNT/C1 recognition sequence useful in a BoNT/C1 substrate can correspond, for example, to a segment of human, mouse or rat SNAP-25, goldfish SNAP-25A or 25B, *Torpedo* SNAP-25, zebrafish SNAP-25, *Drosophila* SNAP-25, leech SNAP-25, or another naturally occurring protein sensitive to cleavage by BoNT/C1. As discussed above in regard to variants of naturally occurring syntaxin sequences, comparison of native SNAP-25 amino acid sequences cleaved by BoNT/C1 reveals significant sequence variability (see Figure 3 and Table B above), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/C1-sensitive SNAP-25 sequence can be tolerated in a BoNT/C1 substrate useful in the invention.

[0134] BoNT/D recognition sequences

[0135] The term "botulinum toxin serotype D recognition sequence" is synonymous with "BoNT/D recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/D under appropriate conditions. A scissile bond cleaved by BoNT/D can be, for example, Lys-Leu.

[0136] A variety of BoNT/D recognition sequences are well known in the art or can be defined by routine methods. A BoNT/D recognition sequence can include, for example, residues 27 to 116; residues 37 to 116; residues 1 to 86; residues 1 to 76; or residues 1 to 69 of rat VAMP-2 (SEQ ID NO: 90; Yamasaki et al., *J. Biol. Chem.* 269:12764-12772 (1994)). Thus, a BoNT/D recognition sequence can include, for example, residues 27 to 69 or residues 37 to 69 of rat VAMP-2 (SEQ ID NO: 90). If desired, a similar BoNT/D recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/D-sensitive VAMP isoform or homolog such as human VAMP-1 or human VAMP-2.

[0137] A BoNT/D recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype D, or can be substantially similar to a segment of a BoNT/D-sensitive protein. As shown in Table D, a variety of naturally occurring proteins sensitive to cleavage by BoNT/D are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; *Torpedo* VAMP-1; *Aplysia* VAMP; squid VAMP; *Drosophila* syb and n-syb; and leech VAMP. Thus, a BoNT/D recognition sequence can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, *Torpedo* VAMP-1, *Aplysia* VAMP, squid VAMP, *Drosophila* syb or n-syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/D. Furthermore, as shown in Table D above, comparison of native VAMP amino acid sequences cleaved by BoNT/D reveals significant sequence variability (see, also, Figure 4), indicating that a variety of amino acid substitutions and modifications

relative to a naturally occurring BoNT/D-sensitive VAMP sequence can be tolerated in a BoNT/D substrate useful in the invention.

[0138] BoNT/E recognition sequences

[0139] As used herein, the term “botulinum toxin serotype E recognition sequence” is synonymous with “BoNT/E recognition sequence” and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/E under appropriate conditions. A scissile bond cleaved by BoNT/E can be, for example, Arg-Ile.

[0140] One skilled in the art appreciates that a BoNT/E recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype E, or can be substantially similar to a segment of a BoNT/E-sensitive protein. In one embodiment, a BoNT/E recognition sequence includes residues 134 to 206 of SEQ ID NO: 2. A variety of naturally occurring proteins sensitive to cleavage by BoNT/E are known in the art and include, for example, human, mouse and rat SNAP-25; mouse SNAP-23; chicken SNAP-25; goldfish SNAP-25A and SNAP-25B; zebrafish SNAP-25; *C. elegans* SNAP-25; and leech SNAP-25 (see Table B). Thus, a BoNT/E recognition sequence can correspond, for example, to a segment of human SNAP-25, mouse SNAP-25, rat SNAP-25, mouse SNAP-23, chicken SNAP-25, goldfish SNAP-25A or 25B, *C. elegans* SNAP-25, leech SNAP-25, or another naturally occurring protein sensitive to cleavage by BoNT/E. Furthermore, as shown in Table B and Figure 3 above, comparison of native SNAP-23 and SNAP-25 amino acid sequences cleaved by BoNT/E reveals that such sequences are not absolutely conserved, indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/E-sensitive SNAP-23 or SNAP-25 sequence can be tolerated in a BoNT/E substrate useful in the invention.

[0141] BoNT/F recognition sequences

[0142] The term “botulinum toxin serotype F recognition sequence,” as used herein, is synonymous with “BoNT/F recognition sequence” and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/F under appropriate conditions. A scissile bond cleaved by BoNT/F can be, for example, Gln-Lys.

[0143] A variety of BoNT/F recognition sequences are well known in the art or can be defined by routine methods. A BoNT/F recognition sequence can include, for example, residues 27 to 116; residues 37 to 116; residues 1 to 86; residues 1 to 76; or residues 1 to 69 of rat VAMP-2 (SEQ ID NO: 90; Yamasaki et al., *supra*, 1994). A BoNT/F recognition sequence also can include, for example, residues 27 to 69 or residues 37 to 69 of rat VAMP-2 (SEQ ID NO: 90). It is understood that a similar BoNT/F recognition sequence can be prepared, if desired, from a corresponding (homologous) segment of another BoNT/F-sensitive VAMP-isoform or homolog such as human VAMP-1 or human VAMP-2.

[0144] A BoNT/F recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype F, or can be substantially similar to a segment of a BoNT/F-sensitive protein. A variety of naturally occurring proteins sensitive to cleavage by BoNT/F are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; *Torpedo* VAMP-1; *Aplysia* VAMP; *Drosophila* syb; and leech VAMP (see Table D). Thus, a BoNT/F recognition sequence can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, *Torpedo* VAMP-1, *Aplysia* VAMP, *Drosophila* syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/F. Furthermore, as shown in Table D above, comparison of native VAMP amino acid sequences cleaved by BoNT/F reveals that such sequences are not absolutely conserved (see, also, Figure 4), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/F-sensitive VAMP sequence can be tolerated in a BoNT/F substrate useful in the invention.

[0145] BoNT/G recognition sequences

[0146] As used herein, the term "botulinum toxin serotype G recognition sequence" is synonymous with "BoNT/G recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a BoNT/G under appropriate conditions. A scissile bond cleaved by BoNT/G can be, for example, Ala-Ala.

[0147] A BoNT/G recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype G, or can be substantially similar to such a BoNT/G-sensitive segment. As illustrated in Table D above, a variety of naturally occurring proteins sensitive to cleavage by BoNT/G are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; and *Torpedo* VAMP-1. Thus, a BoNT/G recognition sequence can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, *Torpedo* VAMP-1, or another naturally occurring protein sensitive to cleavage by BoNT/G. Furthermore, as shown in Table D above, comparison of native VAMP amino acid sequences cleaved by BoNT/G reveals that such sequences are not absolutely conserved (see, also, Figure 4), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/G-sensitive VAMP sequence can be tolerated in a BoNT/G substrate useful in the invention.

[0148] TeNT recognition sequences

[0149] As used herein, the term “tetanus toxin recognition sequence” means a scissile bond together with adjacent or non-adjacent recognition elements, or both, sufficient for detectable proteolysis at the scissile bond by a tetanus toxin under appropriate conditions. A scissile bond cleaved by TeNT can be, for example, Gln-Phe.

[0150] A variety of TeNT recognition sequences are well known in the art or can be defined by routine methods and include sequences corresponding to some or all of the hydrophilic core of a VAMP protein such as human VAMP-1 or human VAMP-2. A TeNT recognition sequence can include, for example, residues 25 to 93 or residues 33 to 94 of human VAMP-2 (SEQ ID NO: 8; Cornille et al., Eur. J. Biochem. 222:173-181 (1994); Foran et al., Biochem. 33: 15365-15374 (1994)); residues 51 to 93 or residues 1 to 86 of rat VAMP-2 (SEQ ID NO: 90; Yamasaki et al., *supra*, 1994); or residues 33 to 94 of human VAMP-1 (SEQ ID NO: 7). A TeNT recognition sequence also can include, for example, residues 25 to 86, residues 33 to 86 or residues 51 to 86 of human VAMP-2 (SEQ ID NO: 8) or rat VAMP-2 (SEQ ID NO: 90). It is understood that a similar TeNT recognition sequence can be prepared, if desired, from a corresponding (homologous) segment of another TeNT-sensitive VAMP isoform or species homolog such as human VAMP-1 or sea urchin or *Aplysia* VAMP.

[0151] Thus, a TeNT recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by tetanus toxin, or can be substantially similar to a segment of a TeNT-sensitive protein. As shown in Table D above, a variety of naturally occurring proteins sensitive to cleavage by TeNT are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-2; rat cellubrevin; chicken VAMP-2; *Torpedo* VAMP-1; sea urchin VAMP; *Aplysia* VAMP; squid VAMP; *C. elegans* VAMP; *Drosophila* n-syb; and leech VAMP. Thus, a TeNT recognition sequence can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-2, rat cellubrevin, chicken VAMP-2, *Torpedo* VAMP-1, sea urchin VAMP, *Aplysia* VAMP, squid VAMP, *C. elegans* VAMP, *Drosophila* n-syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by TeNT. Furthermore, comparison of native VAMP amino acid sequences cleaved by TeNT reveals that such sequences are not absolutely conserved (Table D and Figure 4). This finding indicates that a variety of amino acid substitutions and modifications relative to a naturally occurring TeNT-sensitive VAMP sequence can be tolerated in a TeNT substrate useful in the invention.

[0152] The clostridial toxin substrates of the invention include peptides and peptidomimetics as well as derivatized forms thereof. As used herein, the term “peptidomimetic” is used broadly to mean a peptide-like molecule that is cleaved by the same clostridial toxin as the peptide substrate upon which it is structurally based. Such peptidomimetics include chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, and peptoids, which are peptide-like molecules resulting from oligomeric assembly of N-substituted glycines, and are cleaved by the same clostridial toxin as the peptide substrate upon which the peptidomimetic is derived (see, for example, Goodman and Ro,

Peptidomimetics for Drug Design, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861).

[0153] A variety of peptidomimetics are known in the art including, for example, peptide-like molecules which contain a constrained amino acid, a non-peptide component that mimics peptide secondary structure, or an amide bond isostere. A peptidomimetic that contains a constrained, non-naturally occurring amino acid can include, for example, an α -methylated amino acid; an α,α -dialkyl-glycine or α -aminocycloalkane carboxylic acid; an N^α -C $^\alpha$ cyclized amino acid; an N^α -methylated amino acid; a β - or γ -amino cycloalkane carboxylic acid; an α,β -unsaturated amino acid; a β , β -dimethyl or β -methyl amino acid; a β -substituted-2,3-methano amino acid; an NC $^\delta$ or C $^\alpha$ -C $^\delta$ cyclized amino acid; or a substituted proline or another amino acid mimetic. In addition, a peptidomimetic which mimics peptide secondary structure can contain, for example, a nonpeptidic β -turn mimic; γ -turn mimic; mimic of β -sheet structure; or mimic of helical structure, each of which is well known in the art. A peptidomimetic also can be a peptide-like molecule which contains, for example, an amide bond isostere such as a retro-inverso modification; reduced amide bond; methylenethioether or methylenesulfoxide bond; methylene ether bond; ethylene bond; thioamide bond; trans-olefin or fluoroolefin bond; 1,5-disubstituted tetrazole ring; ketomethylene or fluoroketomethylene bond or another amide isostere. One skilled in the art understands that these and other peptidomimetics are encompassed within the meaning of the term "peptidomimetic" as used herein.

[0154] In any of the methods of the invention, a clostridial toxin substrate can include one or multiple clostridial toxin cleavage sites for the same or different clostridial toxins. In particular embodiments, the invention provides methods that rely on a clostridial toxin substrate which contains a single clostridial toxin cleavage site. In other embodiments, the invention provides methods which rely on a clostridial toxin substrate which contains multiple cleavage sites for the same clostridial toxin. These cleavage sites can be incorporated within identical or different clostridial toxin recognition sequences. As non-limiting examples, a clostridial toxin substrate can have multiple cleavage sites for the same clostridial toxin intervening between the same lanthanide donor complex and acceptor. A clostridial toxin substrate useful in the invention can contain, for example, two or more, three or more, five or more, or ten or more identical or non-identical recognition sequences for the same clostridial toxin. A clostridial toxin substrate useful in the invention also can have, for example, two, three, four, five, six, seven, eight, nine or ten recognition sequences for the same clostridial toxin; the multiple recognition sequences can intervene between the same or different lanthanide donor complex-acceptor pairs.

[0155] A clostridial toxin substrate useful in the invention also can include cleavage sites for different clostridial toxins. In particular embodiments, the invention provides a method that relies on a clostridial toxin substrate which includes multiple cleavage sites for different clostridial toxins all intervening between the same lanthanide donor complex and acceptor. A clostridial toxin substrate can include, for example, cleavage sites for two or more, three or more, or five or more different clostridial toxins all

intervening between the same lanthanide donor complex and acceptor. A clostridial toxin substrate also can incorporate, for example, cleavage sites for two or more, three or more, or five or more different clostridial toxins intervening between at least two lanthanide donor complex-acceptor pairs. In particular embodiments, the invention provides a clostridial toxin substrate having cleavage sites for two, three, four, five, six, seven or eight different clostridial toxins, where the cleavage sites intervene between the same or different lanthanide donor complex-acceptor pairs. In further embodiments, the invention provides a clostridial toxin substrate which has any combination of two, three, four, five, six, seven or eight cleavage sites for any combination of the following clostridial toxins: BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G and TeNT.

[0156] A method of the invention optionally can be performed with multiple substrates. In such a method, a first clostridial toxin substrate is treated with a sample, the first substrate including a first lanthanide donor complex, a first acceptor having an absorbance spectrum which overlaps the emission spectrum of the first lanthanide donor complex, and a first clostridial toxin recognition sequence containing a cleavage site, where the cleavage site intervenes between the first lanthanide donor complex and the first acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the first lanthanide donor complex and the first acceptor. If desired, a second clostridial toxin substrate can be included in the same assay; this second substrate contains a second lanthanide donor complex and second acceptor having an absorbance spectrum which overlaps the emission spectrum of the second lanthanide donor complex, and a second clostridial toxin recognition sequence that is cleaved by a different clostridial toxin than the toxin that cleaves the first clostridial toxin recognition sequence. The lanthanide donor complex-acceptor pair in the second substrate can be the same or different from the lanthanide donor complex-acceptor pair in the first substrate. In this way, a single sample can be simultaneously assayed for the presence of more than one clostridial toxin.

[0157] It is understood that one can use a method of the invention to assay for any combination of clostridial toxins, for example, two, three, four, five, six, seven, eight, or more clostridial toxins. One can assay, for example, any combination of two, three, four, five, six, seven or eight of BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G and TeNT. As an example, an assay can be performed with seven substrates, each of which includes GFP and CS124-DTPA-EMCH-Tb flanking, respectively, a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F or BoNT/G recognition sequence and cleavage site. These substrates can be treated with a sample under conditions suitable for botulinum toxin activity before exciting carbostyryl 124 at 330 nm and monitoring terbium emission at 586 nm. An increase in luminescence intensity at 586 nm (relief of quenching) is indicative of the presence or activity of at least one clostridial toxin. Such an assay can be useful, for example, for assaying food samples or tissue samples for the presence of any botulinum or other clostridial toxin and can be combined, if desired, with one or more subsequent assays for individual clostridial toxins or specific combinations of clostridial toxins.

[0158] It further is understood that a single sample can be assayed for two or more different clostridial toxins using two or more different clostridial toxin substrates, with each substrate containing a different lanthanide donor complex-acceptor pair. The use of multiple substrates can be useful for extending the dynamic range of an assay, as described, for example, in U.S. Patent No. 6,180,340. Those skilled in the art understand that the first antenna in the first lanthanide donor complex can be excited before or after excitation of the second antenna in the second lanthanide donor complex, and that the change in resonance energy transfer of the first substrate can be determined before, at the same time, or after determining resonance energy transfer of the second substrate.

EXAMPLES

[0159] The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I: Preparation of Lanthanide-Based Substrates

[0160] This example describes construction of substrates containing a terbium or other lanthanide ion suitable for assaying for the presence or activity of a clostridial toxin.

A. Construction of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C

[0161] A substrate was prepared as a fusion protein containing green fluorescent protein (GFP), murine SNAP-25 residues 134-206, a polyhistidine affinity tag (6xHis), and a carboxy-terminal cysteine, with several components separated by peptide linkers. As described further below, the substrate was designed such that the GFP and terminal cysteine were present at opposite ends of SNAP-25₍₁₃₄₋₂₀₆₎.

[0162] The SNAP-25 sequence was obtained from pT25FL, a plasmid which contains the full-length mouse SNAP-25 gene inserted in frame with the 3' terminus of the glutathione-S-transferase (GST) gene (GST-SNAP25₍₁₋₂₀₆₎),¹ provided by Professor Dolly (O'Sullivan et al., *J. Biol. Chem.* 274:36897-36904 (1999)). The SNAP-25 sequence from pT25FL was incorporated into a second expression vector, which was designed to have a BirAsp signal sequence for biotinylation and a polyhistidine affinity tag fused to the amino-terminus of residues 134 to 206 of SNAP-25 (BirAsp-polyHis-SNAP25₍₁₃₄₋₂₀₆₎, denoted "BA-SNAP"). The DNA sequence encoding SNAP25₍₁₃₄₋₂₀₆₎ was generated by PCR amplification of the appropriate region of the pT25FL plasmid with PCR primers 5'-GCT AGA TCT CGA GTT AAC CAC TTC CCA GCA TCT TTG-3' (SEQ ID NO: 91; antisense) and 5'-ATC CGG AGG GTA ACA AAC GAT GCC-3' (SEQ ID NO: 92; sense) to produce a SNAP25₍₁₃₄₋₂₀₆₎ PCR product containing a *Bgl* II restriction site (PCR product A).

[0163] The BirAsp sequence, a natural substrate for biotinylation, as well as a polyhistidine affinity tag, were engineered for fusion upstream and in frame with the SNAP25₍₁₃₄₋₂₀₆₎ sequence using synthetic ...

oligonucleotides SEQ ID NOS: 93 and 94, which contained a 20 bp complementary region. These oligonucleotides, 5'-CGA ATT CCG CGG GCC ACC ATG GGA GGA GGA CTG AAC GAC ATC TTC GAG GCT CAA AAG ATC-3' (SEQ ID NO: 93; sense; *Sac* II site underlined) and 5'-TCG TTT GTT ACC CTC CGG ATA TGA TGA TGA TGA TGA TGA TGA TGG GAT CCA TGC CAC TCG ATC TTT TGA GCC TCG AAG A-3' (SEQ ID NO: 94; antisense), were annealed, and the single strand overhangs filled by PCR amplification to yield PCR product B.

[0164] The two double stranded PCR products containing the coding sequences for SNAP25₍₁₃₄₋₂₀₆₎, denoted PCR product A, and BirAsp and polyhistidine, denoted PCR product B, were denatured and annealed. The 20 bp complementary sequence in the two gene fragments is shown in italics in PCR primers SEQ ID NO: 92 and SEQ ID NO: 94. After filling in the overhangs by PCR, the product was amplified with primers SEQ ID NO: 93 and SEQ ID NO: 91. The resulting PCR product, which encoded BirAsp-polyHis-SNAP25₍₁₃₄₋₂₀₆₎ (designated "BA-SNAP"), was digested with *Sac*II and *Bgl*II, and the isolated gene insert ligated into pQBI25fA2 vector digested with *Sac*II and *Bam*HI, to yield plasmid pNTP12 (pQBI25fA2 containing BA-SNAP).

[0165] For expression and purification from *E. coli*, the BA-SNAP gene was transferred into a pTrc99A plasmid (Amersham Pharmacia Biotech). The BA-SNAP gene was isolated from pNTP12 by digestion with *Nco*I and *Xho*I followed by gel purification. Separately, the pTrc99A plasmid was digested with *Nco*I and *Sal*I, and the isolated vector ligated to the BA-SNAP gene to yield plasmid pNTP14 (pTrc99A containing BA-SNAP).

[0166] For cloning of the BA-SNAP gene into plasmid pQE-50, the BA-SNAP fragment was PCR amplified from pNTP14 with primer SEQ ID NO: 91 and primer SEQ ID NO: 95 (5'-CGA AGA TCT GGA GGA CTG AAC GAC ATC TTC-3' (sense; *Bgl* II site underlined)). After digestion with *Bgl*II and *Xho*I, the amplified PCR product was ligated into vector pQE-50, which had been digested with *Bam*HI and *Sal*I. The resulting plasmid, which represents pQE50 containing BA-SNAP, was designated pNTP26.

[0167] A plasmid encoding the green fluorescent protein (GFP) fusion protein substrate was prepared by modifying vector pQBI T7-GFP (Quantum Biotechnologies; Carlsbad, CA) in three phases as described below. First, vector pQBI T7-GFP was PCR-modified to remove the stop codon at the 3' terminus of the GFP-coding sequence and to insert the coding sequence for a portion of the peptide linker separating GFP from the SNAP-25 fragment. Second, a DNA fragment coding for SNAP-25₍₁₃₄₋₂₀₆₎ was PCR amplified from pNTP26 using PCR primers designed to incorporate the coding sequence for the remainder of the peptide linker fused 5' to the SNAP-25₍₁₃₄₋₂₀₆₎ gene and a 6xHis affinity tag fused 3' of the gene. The resultant PCR product was cloned into the modified pQBI vector described above to yield pQBI GFP-SNAP25₍₁₃₄₋₂₀₆₎.

[0168] Plasmid pQBI GFP-SNAP25₍₁₃₄₋₂₀₆₎ was then modified by site-directed mutagenesis to add a cysteine codon at the carboxy-terminus using primer SEQ ID NO: 96 (5'-GATGGTGATGGTGATGACAG CCGCCACCGCCACC-3' (antisense primer, with the added nucleotides underlined) and its reverse complement (sense primer). The resulting plasmid, designated pQBI GFP-SNAP25 (Cys-Stop), is shown in Figure 7A and was used for expression of GFP-SNAP25₍₁₃₄₋₂₀₆₎-6xHis-Cys. The nucleic acid and predicted amino acid sequence for the GFP-SNAP25₍₁₃₄₋₂₀₆₎-6XHis-cysteine construct is shown herein in Figure 7B.

B. Expression and characterization of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C

[0169] The pQBI GFP-SNAP25 (Cys-Stop) expression vector was transformed into *E. coli* BL21(DE3) cells (Novagen; Madison, WI; or Invitrogen; Carlsbad, CA) or into *E. coli* BL21-CodonPlus[®](DE3)-RIL cells (Stratagene) containing the T7 RNA polymerase gene. Transformed cells were selected on LB-ampicillin plates overnight at 37 °C. Single colonies were used to inoculate 1-3 mL starter cultures, which were in turn used to inoculate 0.5 to 1.0 L cultures. The large cultures were grown at 37 °C with shaking until A₅₉₅ reached 0.5-0.6, at which time they were removed from the incubator and were allowed to cool briefly. After induction of protein expression with 1 mM IPTG, GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C substrate was expressed from the pQBI GFP-SNAP25 (Cys-Stop) plasmid overnight with shaking at 16 °C in order to facilitate formation of the GFP fluorophore. Cells from 250 mL aliquots of the expression cultures were collected by centrifugation (30 minutes, 6,000 x g, 4 °C) and stored at -80 °C until needed.

[0170] Substrates were purified at 4 °C by a two-step procedure involving IMAC purification, followed by a de-salting step to remove NaCl and imidazole, typically yielding greater than 150 mg/L of purified substrate as follows. Cell pellets from 250 mL cultures were each resuspended in 7-12 mL Column Binding Buffer (25 mM HEPES, pH 8.0; 500 mM NaCl; 1 mM β-mercaptoethanol; 10 mM imidazole), lysed by sonication (1 minute 40 seconds in 10-second pulses at 38% amplitude), and clarified by centrifugation (16000 rpm, 4 °C, 1 hour). Affinity resin (3-5 mL Talon SuperFlow Co²⁺ per cell pellet) was equilibrated in a glass or disposable column support (Bio-Rad) by rinsing with 4 column volumes of sterile ddH₂O and 4 column volumes of Column Binding Buffer. Clarified lysate was applied to the column in one of two ways: (1) Lysate was added to the resin and batch bound by horizontal incubation for 1 hour with gentle rocking or (2) Lysate was applied to the vertical column and allowed to enter the column slowly by gravity flow. Following batch binding only, the column was righted and the solution drained, collected, and passed over the resin again. In both cases, after the lysate had been applied, the column was washed with 4-5 column volumes of Column Binding Buffer. In some cases, the column was further washed with 1-2 column volumes of Column Wash Buffer (25 mM HEPES, pH 8.0; 500 mM NaCl; 1 mM β-mercaptoethanol; 20 mM imidazole). Protein was eluted with 1.5 to 2.0 column volumes of Column Elution Buffer (25 mM HEPES, pH 8.0; 500 mM NaCl; 1 mM β-mercaptoethanol; 250 mM imidazole), which was collected in fractions of ~1.4 mL. The green fractions were combined, concentrated with a centrifugal filter (10,000 or 30,000 molecular weight cut-off) and desalted by FPLC (BioRad Biologic).

DuoLogic, QuadTec UV-Vis detector) with a HiPrep 26/10 size exclusion column (Pharmacia) and an isocratic mobile phase of chilled Fusion Protein Desalting Buffer (50 mM HEPES, pH 7.4, 4 °C) at a flow rate of 10 mL/minute. Desalted protein was collected as a single fraction, and the concentration determined using a BioRad Protein Assay. The GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C substrate was analyzed by reducing SDS-PAGE. The protein solution was subsequently divided into 500 µL aliquots, flash-frozen with liquid nitrogen and stored at -80 °C. Once defrosted, a working aliquot was stored at 4 °C, protected from light.

C. Labeling with lumiphore CS124-DTPA-EMCH-Tb

[0171] The GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C construct contains a single cysteine residue which is solvent exposed, although there are three buried cysteine residues within GFP which are not available for chemical modification (Selvin, *supra*, 2000; Heyduk, *Curr. Opin. Biotech.* 13:292-296 (2002)). The carboxy-terminal cysteine residue can therefore be selectively labeled using a fluorophore-maleimide at neutral pH. Shown in Figures 8A and 8B, respectively, are the absorption and emission/excitation spectra of purified GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C protein. The concentration of the protein solution was determined to be 2.74mg/ml based on the theoretical molar extinction coefficient of 20250 M⁻¹cm⁻¹ as calculated from the primary sequence of the construct. The molecular weight of the purified GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C protein was confirmed to be about 37,000 using Matrix Assisted Laser Desorption Time of Flight mass spectrometry (MALDI-TOF).

[0172] The lumiphore CS124-DTPA-EMCH-Tb was obtained from Invitrogen Lifetechnologies (Carlsbad, CA), and GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C-CS124-DTPA-EMCH-Tb was produced by derivatizing the carboxy-terminal cysteine of GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C using maleimide chemistry at pH 6.9 in HEPES buffer. Unreacted probe was removed by extensive dialysis in 20 mM HEPES buffer pH 6.9 using a 25 kDa membrane. The absorption and emission spectra of the resulting CS124-DTPA-EMCH-Tb labeled GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C Are Shown In Figures 9A And 9B, Respectively.

EXAMPLE II: Clostridial Toxin Activity Assays Using Lanthanide-Based Substrates

[0173] This Example Describes The Use Of A Lanthanide-Based Substrate For Assaying Bont/A Activity.

[0174] Upon Excitation Of The Sensitizing Group Carbostyryl 124 (CS124) At 330 Nm, Terbium Produces A Long Lifetime Emission In A Series Of Four Prominent Sharp Bands At 490 Nm, 546 Nm, 586 Nm And 622 nm (see Figure 9B). GFP absorbs maximally at 474 nm, with an emission maximum at 507 nm. Energy transfer was observed by monitoring Tb emission at 586 nm. As shown in Figure 10A, there was a notable increase in luminescence intensity during the addition of reduced bulk BoNT-A toxin, indicative of the relief of quenching between the lanthanide donor complex and GFP. Furthermore, the signal-to-noise ratio for the emission process was enhanced by utilizing a gated process to monitor

emission. By opening the emission gate for the emitted light after 200 μ s, all the emission due to spurious fluorescent contaminants with lifetimes much shorter than the 10^1 - 10^2 μ s lifetimes of the lanthanide probe was avoided. As shown in Figure 10B, in which the dotted trace represents gated terbium emission before turnover of substrate and the solid trace represents gated terbium emission after turnover, the resulting gated signal was very clean and contributed to good levels of sensitivity.

[0175] These results indicate that GFP-SNAP25₍₁₃₄₋₂₀₆₎-His6-C can be derivatized with a commercially available lanthanide donor complex such as CS124-DTPA-EMCH-Tb to produce a clostridial toxin substrate which exhibits luminescence resonance energy transfer between the lanthanide donor complex and an acceptor such as GFP. The relief of quenching upon addition of BoNT/A reduced toxin was indicative of the activity of BoNT/A.

[0176] All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

[0177] Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A clostridial toxin substrate, comprising:
 - (a) a lanthanide donor complex;
 - (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said lanthanide donor complex; and
 - (c) a clostridial toxin recognition sequence comprising a cleavage site,wherein said cleavage site intervenes between said lanthanide donor complex and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said lanthanide donor complex and said acceptor.
2. The substrate of claim 1, wherein said lanthanide donor complex has a fluorescence lifetime of at least 500 μ s.
3. The substrate of claim 1, wherein said lanthanide donor complex has a fluorescence quantum yield of at least 0.05.
4. The substrate of claim 1, wherein said lanthanide donor complex has a fluorescence quantum yield of at least 0.5.
5. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide ion selected from the group of a terbium ion, europium ion, samarium ion and dysprosium ion.
6. The substrate of claim 5, wherein said lanthanide ion is a terbium ion.
7. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide-binding site which is a peptide or peptidomimetic.
8. The substrate of claim 7, wherein said lanthanide-binding site comprises the coordination site of an EF hand motif.
9. The substrate of claim 8, wherein said lanthanide-binding site comprises an EF hand motif.
10. The substrate of claim 7, wherein said lanthanide-binding site comprises a thiol-reactive chelator.
11. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide-binding site which comprises diethylenetriaminepentacetic acid (DTPA).
12. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide-binding site which is selected from the group of a β -diketone chelate, polyaminopolycarboxylic acid chelate, calixarene chelate, polyphenol, DOTA, pyridine and polypyridine.
13. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide-binding site which is selected from the group trisbipyridine (TBP) cryptate; trisbipyridine tetracarboxylate (TBP4COOH) cryptate; trisbipyridine pentacarboxylate (TBP5COOH) cryptate; and pyridine bipyridine tetracarboxylate (PBP4COOH).
14. The substrate of claim 1, wherein said lanthanide donor complex comprises a lanthanide-binding site which has an affinity for a lanthanide ion of at least 5 μ M.
15. The substrate of claim 1, 6 or 8, wherein said lanthanide donor complex comprises an antenna which is a tryptophan residue.

16. The substrate of claim 1, wherein said lanthanide donor complex comprises an antenna which is selected from the group carbostyryl124 (CS124), tryptophan and 2-hydroxyisophthalamide.
17. The substrate of claim 16, wherein said antenna is carbostyryl124 (CS124).
18. The substrate of claim 11, wherein said lanthanide donor complex is CS124-DTPA-EMCH-Tb.
19. The substrate of claim 1, wherein said acceptor is an acceptor fluorophore.
20. The substrate of claim 1 or claim 18, wherein said acceptor is selected from the group green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP).
21. The substrate of claim 20, wherein said acceptor is GFP.
22. The substrate of claim 1, wherein said acceptor is a non-fluorescent acceptor.
23. The substrate of claim 22, wherein said non-fluorescent acceptor is a heme protein.
24. The substrate of claim 1, comprising a botulinum toxin recognition sequence.
25. The substrate of claim 24, wherein said recognition sequence is a BoNT/A recognition sequence.
26. The substrate of claim 25, wherein said BoNT/A recognition sequences comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Gln-Arg, or a peptidomimetic thereof.
27. The substrate of claim 24, wherein said recognition sequence is a BoNT/B recognition sequence.
28. The substrate of claim 27, wherein said BoNT/B recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.
29. The substrate of claim 24, wherein said recognition sequence is a BoNT/C1 recognition sequence.
30. The substrate of claim 29, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of syntaxin, said six consecutive residues comprising Lys-Ala, or a peptidomimetic thereof.
31. The substrate of claim 29, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ala, or a peptidomimetic thereof.
32. The substrate of claim 24, wherein said recognition sequence is a BoNT/D recognition sequence.
33. The substrate of claim 32, wherein said BoNT/D recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Lys-Leu, or a peptidomimetic thereof.
34. The substrate of claim 24, wherein said recognition sequence is a BoNT/E recognition sequence.
35. The substrate of claim 34, wherein said BoNT/E recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ile, or a peptidomimetic thereof.
36. The substrate of claim 24, wherein said recognition sequence is a BoNT/F recognition sequence.

37. The substrate of claim 36, wherein said BoNT/F recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Lys, or a peptidomimetic thereof.
38. The substrate of claim 24, wherein said recognition sequence is a BoNT/G recognition sequence.
39. The substrate of claim 38, wherein said BoNT/G recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Ala-Ala, or a peptidomimetic thereof.
40. The substrate of claim 1, wherein said recognition sequence is a TeNT recognition sequence.
41. The substrate of claim 40, wherein said TeNT recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.
42. The substrate of claim 1, 6 or 7, which is a peptide or peptidomimetic having at most 300 residues.
43. The substrate of claim 1, 6 or 7, which is a peptide or peptidomimetic having at most 150 residues.
44. The substrate of claim 1, wherein said substrate can be cleaved with an activity of at least 1 nanomole/minute/milligram toxin.
45. The substrate of claim 1, wherein said substrate can be cleaved with an activity of at least 20 nanomoles/minute/milligram toxin.
46. The substrate of claim 1, wherein said substrate can be cleaved with an activity of at least 100 nanomoles/minute/milligram toxin.
47. A method of determining the presence or activity of a clostridial toxin, comprising the steps of:
- (a) treating with a sample, under conditions suitable for clostridial toxin protease activity, a clostridial toxin substrate comprising
 - (i) a lanthanide donor complex;
 - (ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of said lanthanide donor complex; and
 - (iii) a clostridial toxin recognition sequence comprising a cleavage site,wherein said cleavage site intervenes between said lanthanide donor complex and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said lanthanide donor complex and said acceptor;
 - (b) exciting an antenna of said lanthanide donor complex; and
 - (c) determining resonance energy transfer of said treated substrate relative to a control substrate,
- wherein a difference in resonance energy transfer of said treated substrate as compared to said control substrate is indicative of the presence or activity of said clostridial toxin.
48. The method of claim 47, wherein said lanthanide donor complex has a fluorescence lifetime of at least 500 μ s.
49. The method of claim 47, wherein said lanthanide donor complex has a fluorescence quantum yield of at least 0.05.

50. The method of claim 47, wherein said lanthanide donor complex has a fluorescence quantum yield of at least 0.5.
51. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide ion selected from the group of a terbium ion, europium ion, samarium ion and dysprosium ion.
52. The method of claim 51, wherein said lanthanide ion is a terbium ion.
53. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which is a peptide or peptidomimetic.
54. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which comprises the coordination site of an EF hand motif.
55. The method of claim 54, wherein said lanthanide donor complex comprises a lanthanide-binding site which comprises an EF hand motif.
56. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which comprises a thiol-reactive chelator.
57. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which comprises diethylenetriaminepentacetic acid (DTPA).
58. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which is selected from the group β -diketone chelate, polyaminopolycarboxylic acid chelate, calixarene chelate, polyphenol, DOTA, pyridine and polypyridine.
59. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which is selected from the group trisbipyridine (TBP) cryptate; trisbipyridine tetracarboxylate (TBP4COOH) cryptate; trisbipyridine pentacarboxylate (TBP5COOH) cryptate; and pyridine bipyridine tetracarboxylate (PBP4COOH).
60. The method of claim 47, wherein said lanthanide donor complex comprises a lanthanide-binding site which has an affinity for said lanthanide ion of at least 5 μ M.
61. The method of claim 47, 52 or 54, wherein said lanthanide donor complex comprises an antenna which is a tryptophan residue.
62. The method of claim 47, wherein said lanthanide donor complex comprises an antenna which is selected from the group carbostyryl-124, tryptophan, and 2-hydroxyisophthalamide.
63. The method of claim 62, wherein said antenna is carbostyryl124 (CS124).
64. The method of claim 57, wherein said lanthanide donor complex is CS124-DTPA-EMCH-Tb.
65. The method of claim 47, wherein said acceptor is an acceptor fluorophore.
66. The method of claim 65, wherein said acceptor fluorophore is a fluorescent protein.
67. The method of claim 47 or claim 64, wherein said acceptor is selected from the group of a green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP).
68. The method of claim 67, wherein said acceptor is GFP.
69. The method of claim 47, wherein said acceptor is a non-fluorescent acceptor.
70. The method of claim 69, wherein said non-fluorescent acceptor is a heme protein.

71. The method of claim 47, wherein said clostridial toxin substrate comprises a botulinum toxin recognition sequence.
72. The method of claim 71, wherein said recognition sequence is a BoNT/A recognition sequence.
73. The method of claim 72, wherein said BoNT/A recognition sequences comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Gln-Arg, or a peptidomimetic thereof.
74. The method of claim 71, wherein said recognition sequence is a BoNT/B recognition sequence.
75. The method of claim 74, wherein said BoNT/B recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.
76. The method of claim 71, wherein said recognition sequence is a BoNT/C1 recognition sequence.
77. The method of claim 76, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of syntaxin, said six consecutive residues comprising Lys-Ala, or a peptidomimetic thereof.
78. The method of claim 76, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ala, or a peptidomimetic thereof.
79. The method of claim 71, wherein said recognition sequence is a BoNT/D recognition sequence.
80. The method of claim 79, wherein said BoNT/D recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Lys-Leu, or a peptidomimetic thereof.
81. The method of claim 71, wherein said recognition sequence is a BoNT/E recognition sequence.
82. The method of claim 81, wherein said BoNT/E recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ile, or a peptidomimetic thereof.
83. The method of claim 71, wherein said recognition sequence is a BoNT/F recognition sequence.
84. The method of claim 83, wherein said BoNT/F recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Lys, or a peptidomimetic thereof.
85. The method of claim 71, wherein said recognition sequence is a BoNT/G recognition sequence.
86. The method of claim 85, wherein said BoNT/G recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Ala-Ala, or a peptidomimetic thereof.
87. The method of claim 47, wherein clostridial toxin substrate comprises a TeNT recognition sequence.
88. The method of claim 87, wherein said TeNT recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.
89. The method of claim 47, 52 or 53, wherein said substrate is a peptide or peptidomimetic having at most 300 residues.

90. The method of claim 47, 52 or 53, wherein said substrate is a peptide or peptidomimetic having at most 150 residues.
91. The method of claim 47, wherein said substrate can be cleaved with an activity of at least 1 nanomole/minute/milligram toxin.
92. The method of claim 47, wherein said substrate can be cleaved with an activity of at least 20 nanomoles/minute/milligram toxin.
93. The method of claim 47, wherein said substrate can be cleaved with an activity of at least 100 nanomoles/minute/milligram toxin.
94. The method of claim 47, wherein said sample is a crude cell lysate.
95. The method of claim 47, wherein said sample is isolated clostridial toxin.
96. The method of claim 95, wherein said isolated clostridial toxin is isolated clostridial toxin light chain.
97. The method of claim 47, wherein said sample is a formulated clostridial toxin product.
98. The method of claim 97, wherein said formulated clostridial toxin product is a BoNT/A, BoNT/B or BoNT/E toxin product.
99. The method of claim 98, wherein said formulated product is formulated BoNT/A toxin product.
100. A nucleic acid molecule, comprising a nucleotide sequence encoding a clostridial toxin substrate, said clostridial toxin substrate comprising:
 - (a) together with a lanthanide ion, a lanthanide donor complex;
 - (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said lanthanide donor complex; and
 - (c) a clostridial toxin recognition sequence comprising a cleavage site,wherein said cleavage site intervenes between said lanthanide donor complex and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said lanthanide donor complex and said acceptor.
101. The nucleic acid molecule of claim 100, wherein lanthanide donor complex comprises a lanthanide-binding site which comprises the coordination site of an EF hand motif.
102. The nucleic acid molecule of claim 101, wherein said lanthanide-binding site comprises an EF hand motif.
103. The nucleic acid molecule of claim 100 or claim 101, wherein said lanthanide donor complex comprises an antenna which is a tryptophan residue.
104. The nucleic acid molecule of claim 100 or claim 101, wherein said acceptor is an acceptor fluorophore.
105. The nucleic acid molecule of claim 104, wherein said acceptor fluorophore is a fluorescent protein.
106. The nucleic acid molecule of claim 104, wherein said acceptor fluorophore is selected from the group of a green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP) and red fluorescent protein (RFP).
107. The nucleic acid molecule of claim 106, wherein said acceptor fluorophore is GFP.

108. The nucleic acid molecule of claim 100, wherein said acceptor is a non-fluorescent acceptor.
109. The nucleic acid molecule of claim 108, wherein said non-fluorescent acceptor is a heme protein.
110. The nucleic acid molecule of claim 100, wherein said recognition sequence is a botulinum toxin recognition sequence.
111. The nucleic acid molecule of claim 110, wherein said botulinum toxin recognition sequence is a BoNT/A recognition sequence.
112. The nucleic acid molecule of claim 111, wherein said BoNT/A recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Gln-Arg.
113. The nucleic acid molecule of claim 112, wherein said BoNT/A recognition sequence comprises residues 134 to 206 of SEQ ID NO: 2.
114. The nucleic acid molecule of claim 110, wherein said recognition sequence is a BoNT/B recognition sequence.
115. The nucleic acid molecule of claim 114, wherein said BoNT/B recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe.
116. The nucleic acid molecule of claim 110, comprising a BoNT/C1 substrate comprising a BoNT/C1 recognition sequence.
117. The nucleic acid molecule of claim 116, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of syntaxin, said six consecutive residues comprising Lys-Ala.
118. The nucleic acid molecule of claim 116, wherein said BoNT/C1 recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ala.
119. The nucleic acid molecule of claim 110, said recognition sequence is a BoNT/D recognition sequence.
120. The nucleic acid molecule of claim 119, wherein said BoNT/D recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Lys-Leu.
121. The nucleic acid molecule of claim 110, wherein said recognition sequence is a BoNT/E recognition sequence.
122. The nucleic acid molecule of claim 121, wherein said BoNT/E recognition sequence comprises at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ile.
123. The nucleic acid molecule of claim 110, comprising a BoNT/F substrate comprising a BoNT/F recognition sequence.
124. The nucleic acid molecule of claim 123, wherein said BoNT/F recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Lys.
125. The nucleic acid molecule of claim 110, comprising a BoNT/G substrate comprising a BoNT/G recognition sequence.
126. The nucleic acid molecule of claim 125, wherein said BoNT/G recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Ala-Ala.
127. The nucleic acid molecule of claim 100, wherein said recognition sequence is a TeNT recognition sequence.

128. The nucleic acid molecule of claim 127, wherein said TeNT recognition sequence comprises at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe.
129. The nucleic acid molecule of claim 100, wherein said nucleotide sequence encodes a clostridial toxin substrate having at most 300 residues.
130. The nucleic acid molecule of claim 100, wherein said nucleotide sequence encodes a clostridial toxin substrate having at most 150 residues.

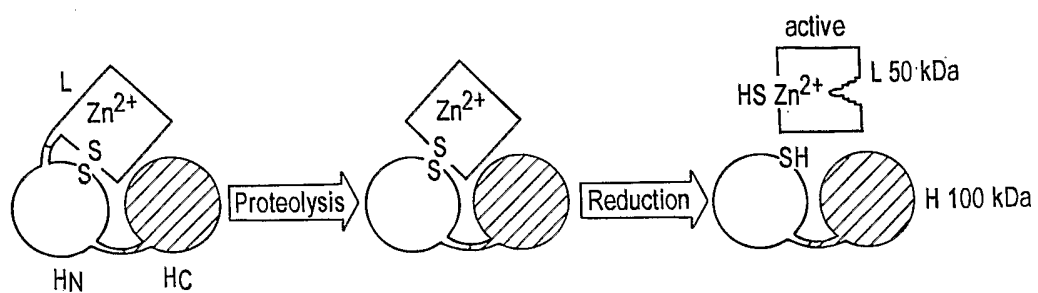
FIG. 1

FIG. 2

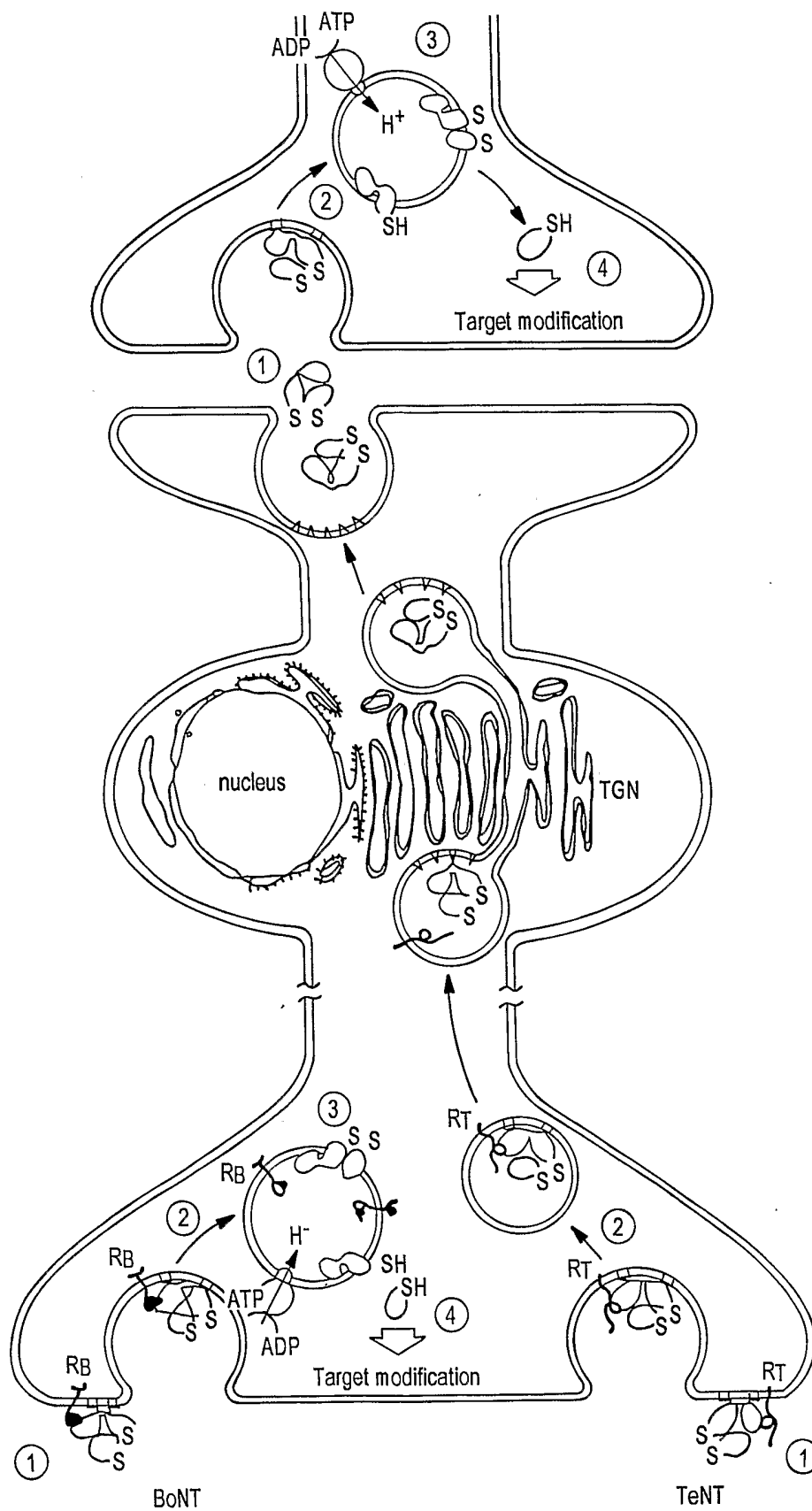


FIG. 3

SNAP-25 Human	(1)	1	-----MADADMRNELEEMQRRADQIADDESLESTRMLQIVEESKDAGIRTLVMDDEQGEOLERTIEEGMDQIN	75
SNAP-25 Mouse	(1)	-----MADADMRNELEEMQRRADQIADDESLESTRMLQIVEESKDAGIRTLVMDDEQGEOLERTIEEGMDQIN		
SNAP-25 Drosophila	(1)	MPADPSEEVAPQVPEKTELEELQINAGVADDESLESTRMLALPEESKEAGIRTLVMDDEQGEOLERTIEEGMDQIN		
SNAP-25 Goldfish	(1)	-----MADADMRNELEEMQRRADQIADDESLESTRMLQIVEESKDAGIRTLVMDDEQGEOLERTIEEGMDQIN		
SNAP-25 Sea Urchin	(1)	-----MEDQNMNRSELEELQVQSNMQIDESLESTRMSQMAEESQYGIKTLVMDDEQGEOLERTIEEGMDQIN		
SNAP-25 Chicken	(1)	-----MADADMRNELEEMQRRADQIADDESLESTRMLQIVEESKDAGIRTLVMDDEQGEOLERTIEEGMDQIN		
SNAP-25 Human	(69)	76	KDMKEAEKNTLDLGNFCGLCVCPCN--KLKSSDAKKKAAGNNQDGVVASQPARVVDEREQMAISGGFIRRVTN	150
SNAP-25 Mouse	(69)	KDMKEAEKNTLDLGNFCGLCVCPCN--KLKSSDAKKKAAGNNQDGVVASQPARVVDEREQMAISGGFIRRVTN		
SNAP-25 Drosophila	(76)	ADMREAEKNTSGMEKCCGICVLPCKMSOSEKEDDg---TWKGNDDGKVANNQFQRAVVDEREQMAISGGFIRRVTN		
SNAP-25 Goldfish	(69)	KDMKEAEKNTLDLGNLCGLCFCPCN--KLKSG--GQSWANNQDGVVSSQPARVVDEREQMAISGGFIRRVTN		
SNAP-25 Sea Urchin	(71)	TPMRBAEKNTLTELKCCGICVCPCPKKLGNFEKGDYKKTWKGNDGKVNSHQFQRAVVDEREQMAISGGFIRRVTN		
SNAP-25 Chicken	(69)	QDMKEAEKNTLDLGNCCGIFICPCN--KLKSSDAKKKAAGNNQDGVVASQPARVVDEREQMAISGGFIRRVTN		
SNAP-25 Human	(140)	151	DARENEMDENTLEQVSGTIGNLRHMAIDMGNEIDTONRQIDRIMEKADSNKIRIDEAN--QRAITKMLSSG-----	225
SNAP-25 Mouse	(140)	DARENEMDENTLEQVSGTIGNLRHMAIDMGNEIDTONRQIDRIMEKADSNKIRIDEAN--QRAITKMLSSG-----		
SNAP-25 Drosophila	(148)	DAREEMENMGQVNTTMTGNLRHMAIDMGSELENQNRQIDRINRKESNEARIVAVAN--QRAHQLK-----		
SNAP-25 Goldfish	(137)	DARENEMDENTLEQVSGTIGNLRHMAIDMGNEIDTONRQIDRIMADSNKIRIDEAN--QRAITKMLSSG-----		
SNAP-25 Sea Urchin	(146)	DAREEMDENTLPVYSILVGNLRHMAIDMGSEIGANQSGQZGRITSKAESNEGRINSAD--KRAKNTLFNK-----		
SNAP-25 Chicken	(140)	DARENEMDENTLEQVSGTIGNLRHMAIDMGNEIDTONRQIDRIMEKLPKPGIMKPTSVQQRCSAVVAKCSKVHFL		
SNAP-25 Human	(207)	226	-----	260
SNAP-25 Mouse	(207)	-----		
SNAP-25 Drosophila	(213)	-----		
SNAP-25 Goldfish	(204)	-----		
SNAP-25 Urchin	(213)	-----		
SNAP-25 Chicken	(215)	IMLSQRAVPSCFYHGYILGLHTCTYQPHCKCPV		

FIG. 4

VAMP-1 HUMAN	(1)	1	MSAPAPPHLEGTEGTAPELGGPEPPENMTSNRRLOQTQAQVEHVVDLRRVNVDKVLERDQKLSLDDRADALQA	75
VAMP-2 HUMAN	(1)	---	MSATAATAPPAAPAGEGPPAPPNTLSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSLDDRADALQA	
VAMP-2 MOUSE	(1)	---	MSATAATVPAPAPAGEGPPAPPNTLSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSLDDRADALQA	
VAMP Bovine	(1)	---	MSATAATAPPAAPAGEGPPAPPNTLSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSLDDRADALQA	
VAMP-2 Frog	(1)	---	MSAPPAAGPPAPAPPEPPHNTLSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLSLDDRADALQA	
VAMP Sea Urchin	(1)	-----	MAAPPPHQPAPSNKRLQQTQAQVDEVVDIMRVNVDKVLERDQALSTLDDRADALQA	
VAMP-1 HUMAN	(75)	76	GASQFEESAAKLKKRYWWKNCCKWMTMTGALICAITVVVTVLVEHETL---	123
VAMP-2 HUMAN	(73)	---	GASQFEESAAKLKKRYWWKNLKMWIILGVICAIILIIIVYFSS---	
VAMP-2 MOUSE	(73)	---	GASQFEESAAKLKKRYWWKNLKMWIILGVICAIILIIIVYFST---	
VAMP Bovine	(73)	---	GASQFEESAAKLKKRYWWKNLKMWIILGVICAIILIIIVYFSS---	
VAMP-2 Frog	(71)	---	GASQFEESAAKLKKRYWWKNCCKWMIIMGVICAIILIIIVYFST---	
VAMP Sea Urchin	(57)	---	GASQFETNAQKLKKRYWWKNCCKWMIILATLITVLLIIIVAVQSQKK	

FIG. 5

Syntaxin 1A human	(1)	1	MDRIQELRIAK-DSDDDDVAIVTD-RDEWDEFFEQVEIRGFIDKIAENVEEVRKHSATILASPNPDEKTK	75
Syntaxin 1B2 human	(1)		MKDRIQELRSAK-DSDDDEEV-WHVD-RDEWDEFFEQVEEIRGCTLEKLSDEYEQVKQHSATILANPDEKTK	
Syntaxin 1A mouse	(1)		MKDRIQELRIAK-DSDDDDVAIVTD-RDEWDEFFEQVEEIRGFIIDKIAENVEEVRKHSATILASPNPDEKTK	
Syntaxin 1a drosophila	(1)		MKDRILAAHAAQSDDEEETEVAVNVDGHPSTYNDFFAQVEEIRGMIDKQDNVEEVRKHSATILASPNPDEKTK	
Syntaxin A C. elegans	(1)		MKDRLSALKAAQSEDEQDDMHMDTG-NAQYVEEVEFEQVEEIRGSDPILANNVEEVRKHSATILASPNVDQKTK	
Syntaxin Sea urchin	(1)		MKDRIGSLKRNE-EDVDGPEVAVNVE-SEKVEEVEFEQVEEIRGSDPILANNVDKISKNVDVKKKHSATILASPNVDKTK	
Syntaxin 1A human	(73)	76	EELEELMSDIKKTANKVRSKLRKSIQEOSIEQEEGINRSSADLRIRKTOHSTLSRKFEVEMSEYNATQSDYRERCKG	150
Syntaxin 1B2 human	(72)		CELELDIADIKKTANKVRSKLRKPIEQOSIEQEEGINRSSADLRIRKTOHSTLSRKFEVEMTEYNATQSDYRERCKD	
Syntaxin 1A mouse	(73)		EELEELMSDIKKTANKVRSKLRKSIQEOSIEQEEGINRSSADLRIRKTOHSTLSRKFEVEMSEYNATQSDYRERCKG	
Syntaxin 1a drosophila	(76)		CELELDIADIKKTANKVRSKLRKPIEQOSIEQEEGINRSSADLRIRKTOHSTLSRKFEVEMTEYNATQSDYRERCKG	
Syntaxin A C. elegans	(75)		EELEELMAVIRKANKVRSKLRKLEENALDHEQG-AGNADLRIRKTOHSTLSRKFEVEMDYNKTOQSDYRERCKG	
Syntaxin Sea urchin	(73)		DELEELMSDIKKTANKVRSKLRKMEQOSIEQEESSAKMNSADLRIRKTOHSTLSRKFEVEMDYNSTQSDYRERCKG	
Syntaxin 1A human	(148)	151	RIQROLETGRTTISEELEDMLESNGNPAIFPSGIIMDSISISKQALSEIEIRHSEITIKLENISIRELHDMFMDAMTL	225
Syntaxin 1B2 human	(147)		RIQROLETGRTTISEELEDMLESNGKLAIFDDIKMDSQMTKQALNEIEIRHNEI IKLETSIRELHDMFMDAMTL	
Syntaxin 1A mouse	(148)		RIQROLETGRTTISEELEDMLESNGNPAIFPSGIIMDSISISKQALSEIEIRHSEI IKLETSIRELHDMFMDAMTL	
Syntaxin 1a drosophila	(151)		RIQROLETGRTTISEELEDMLESNGNPAIFPSGIIMDSISISKQALSEIEIRHSEI IKLETSIRELHDMFMDAMTL	
Syntaxin A C. elegans	(149)		RIQROLDIAEKQVGEDELEEMLESNGNPGVFTGSIITDPOAKQILADIEARHNDIMKLESISIRELHDMFMDAMTL	
Syntaxin Sea urchin	(148)		RIQROLETGRTTISEELEDMLESNGNPAIFPSGIIMDPOAKQILADIEARHNDIMKLESISIRELHDMFMDAMTL	
Syntaxin 1A human	(223)	226	VESQGEMIDRIEYVNEHAVDYVERAVSDITKAVKYQSKARRKKIMITICCVLTGIVJASIVGGIEA--	293
Syntaxin 1B2 human	(222)		VESQGEMIDRIEYVNEHSDYVERAVSDITKAVKYQSKARRKKIMITICCVLTGIVJASIVGGIEA--	
Syntaxin 1A mouse	(223)		VESQGEMIDRIEYVNEHAVDYVERAVSDITKAVKYQSKARRKKIMITICCVLTGIVJASIVGGIEA--	
Syntaxin 1a drosophila	(226)		VESQGEMIDRIEYVNEHAVDYVQATQPTKALIKYQSKARRKKIMITIGTIVGILJASIVSSYEM--	
Syntaxin A C. elegans	(224)		VESQGEMIDRIEYVNEHAKEMDRAVADPTKAVDYQSKARRKKIQLIVTGVLITGILIFILFYAKVL	
Syntaxin Sea urchin	(223)		VESQGEMIDRIEYVNEQSDYVEINAKMDITKAVKYQSKARRKKIYALICGVALGILVLLIIVLA--	

FIG. 6

CANONICAL

----- helix E -----										----- loop -----										----- helix F -----									
1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	2	2	2	2	2	2	2	2
E	n	*	*	n	n	*	*	n	X	*	Y	*	Z	G	#	I	-X	*	*	-Z	n	*	n	n	*	n	*	*	(n)

FIG. 7A

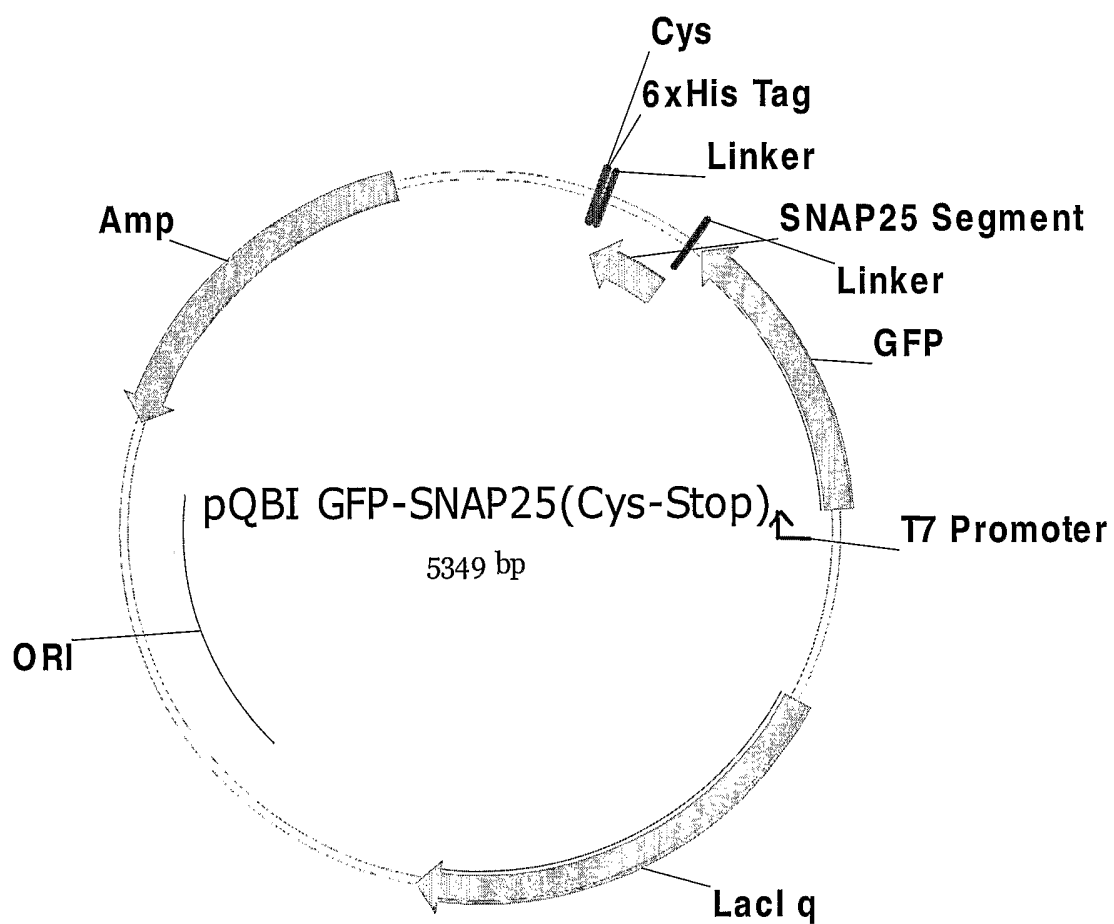


FIG. 7B

```

                                GFP
~~~~~
M A S K G E E L F T G V V P I L V E L D G
ATGGCTAGC AAAGGAGAA GAACTCTTC ACTGGAGTT GTCCCAATT CTTGTTGAA TTAGATGGT
                                GFP
~~~~~
D V N G H K F S V S G E G E G D A T Y G K
GATGTTAAC GGCCACAAG TTCTCTGTC AGTGGAGAG GGTGAAGGT GATGCAACA TACGGAAAA
                                GFP
~~~~~
L T L K F I C T T G K L P V P W P T L V T
CTTACCTTG AAGTTCATC TGCACTACT GGCAAACTG CCTGTTCCA TGGCCAACA CTAGTCACT
                                GFP
~~~~~
T L C Y G V Q C F S R Y P D H M K R H D F
ACTCTGTGC TATGGTGTG CAATGCTTT TCAAGATAC CCGGATCAT ATGAAACGG CATGACFTT
                                GFP
~~~~~
F K S A M P E G Y V Q E R T I F F K D D G
TTCAAGAGT GCCATGCC GAAGGTTAT GTACAGGAA AGGACCATC TTCTTCAA GATGACGGC
                                GFP
~~~~~
N Y K T R A E V K F E G D T L V N R I E L
AACTACAAG ACACGTGCT GAAGTCAAG TTTGAAGGT GATACCCTT GTTAATAGA ATCGAGTTA
                                GFP
~~~~~
K G I D F K E D G N I L G H K L E Y N Y N
AAAGGTATT GACTTCAAG GAAGATGGC AACATTCTG GGACACAAA TTGGAATAC AACTATAAC
                                GFP
~~~~~
S H N V Y I M A D K Q K N G I K V N F K T
TCACACAAT GTATACATC ATGGCAGAC AAACAAAAG AATGGAATC AAAGTGAAC TTCAAGACC
                                GFP
~~~~~
R H N I E D G S V Q L A D H Y Q Q N T P I
CGCCACAAC ATTGAAGAT GGAAGCGTT CAACTAGCA GACCATTAT CAACAAAAT ACTCCAATT
                                GFP
~~~~~
G D G P V L L P D N H Y L S T Q S A L S K
GGCGATGGC CCTGTCCTT TTACCAGAC AACCATTAC CTGTCCACA CAATCTGCC CTTTCGAAA
                                GFP
~~~~~
D P N E K R D H M V L L E F V T A A G I T
GATCCCAAC GAAAAGAGA GACCACATG GTCCTTCTT GAGTTTGTA ACAGCTGCT GGGATTACA
                                Linker
~~~~~
                                GFP
~~~~~
H G M D E L Y N G G A G S G A G G G G I R
CATGGCATG GATGAACTG TACAACGGC GGTGCAGGA TCCGGTGCG GGTGGCGGT GGCATCCGG
                                SNAP25 (134-206)
~~~~~
R V T N D A R E N E M D E N L E Q V S G I
AGGGTAACA AACGATGCC CGGGAAAAT GAGATGGAT GAGAACCTG GAGCAGGTG AGCGGCATC
                                SNAP25 (134-206)
~~~~~
I G N L R H M A L D M G N E I D T Q N R Q
ATCGGAAAC CTCCGCCAT ATGGCTCTA GACATGGGC AATGAGATT GACACCCAG AATCGCCAG
                                SNAP25 (134-206)
~~~~~
I D R I M E K A D S N K T R I D E A N Q R
ATCGACAGG ATCATGGAG AAGGCTGAT TCCAACAAA ACCAGAATT GATGAAGCC AACCACCT
                                Linker
                                Cys
                                6xHis Tag
~~~~~
SNAP25 (134-206) ~~~~~
A T K M L G S G G G G G H H H H H H C *
GCAACAAAAG ATGCTGGGA AGTGGTGGC GGTGGCGGC CATCACCAT CACCATCAC TGCTAA

```

FIG. 8A

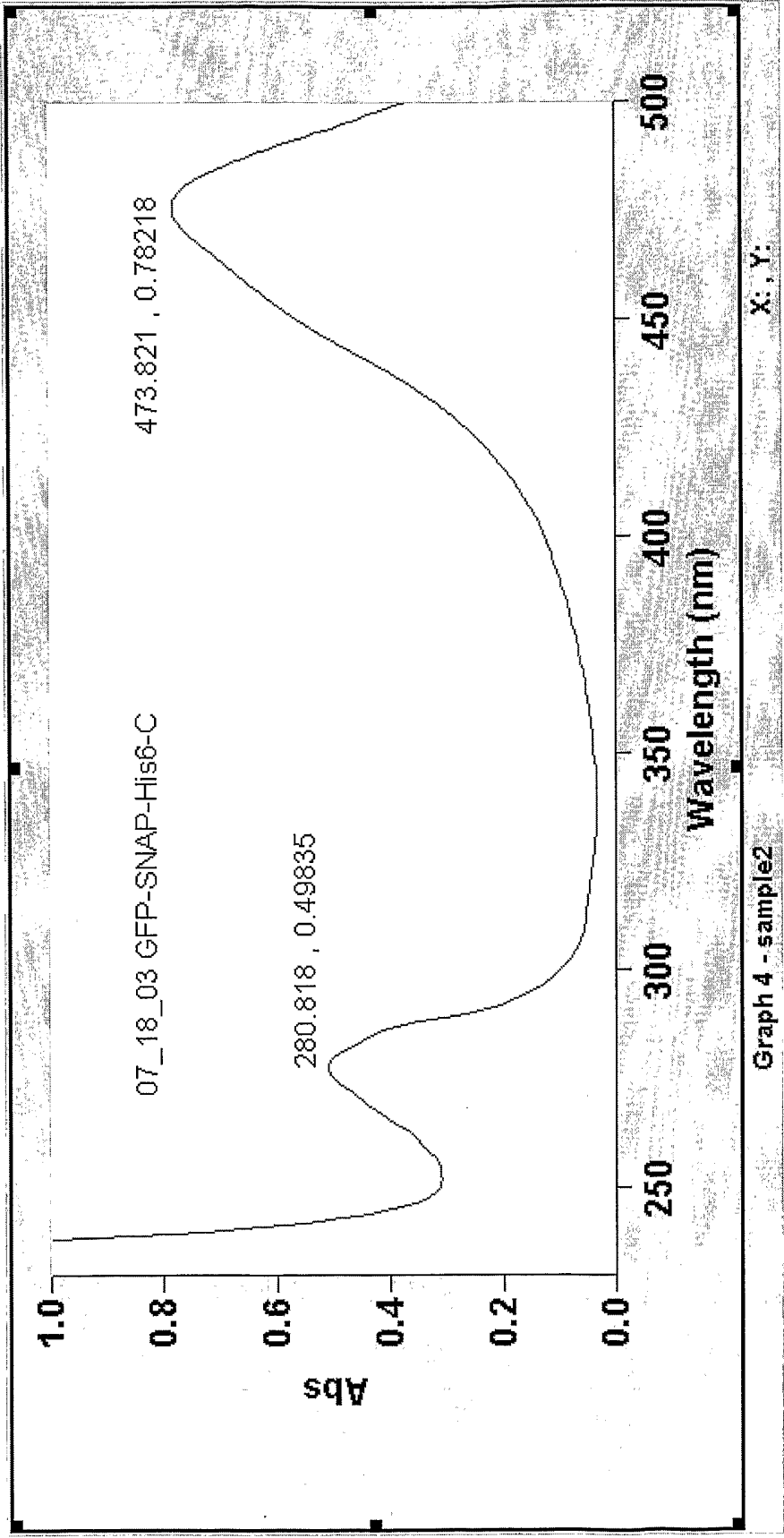


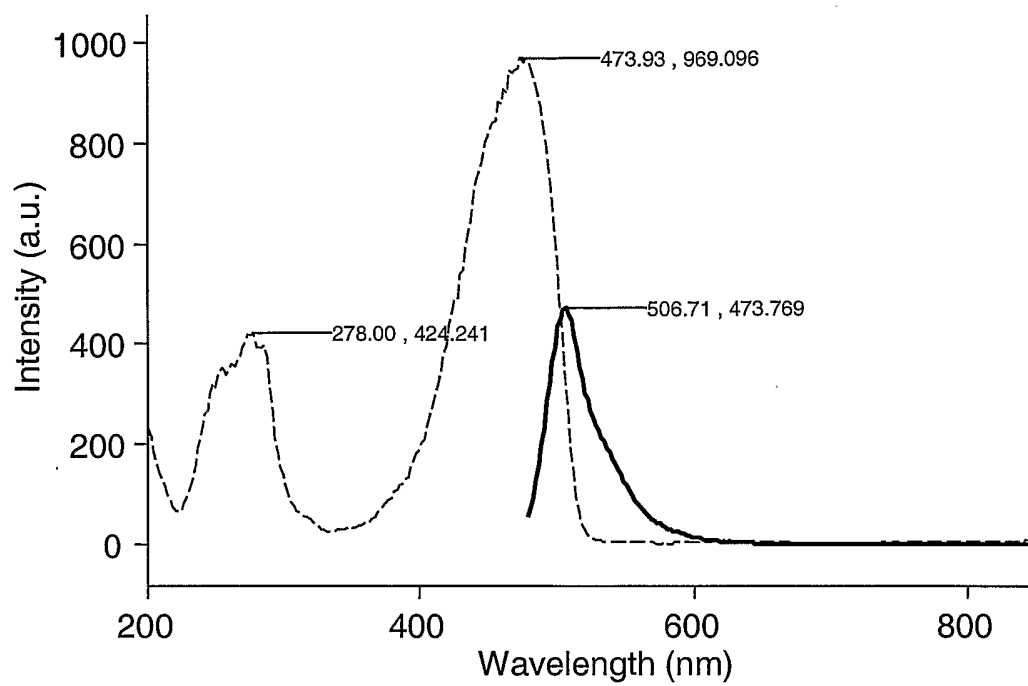
FIG. 8B

FIG. 9A

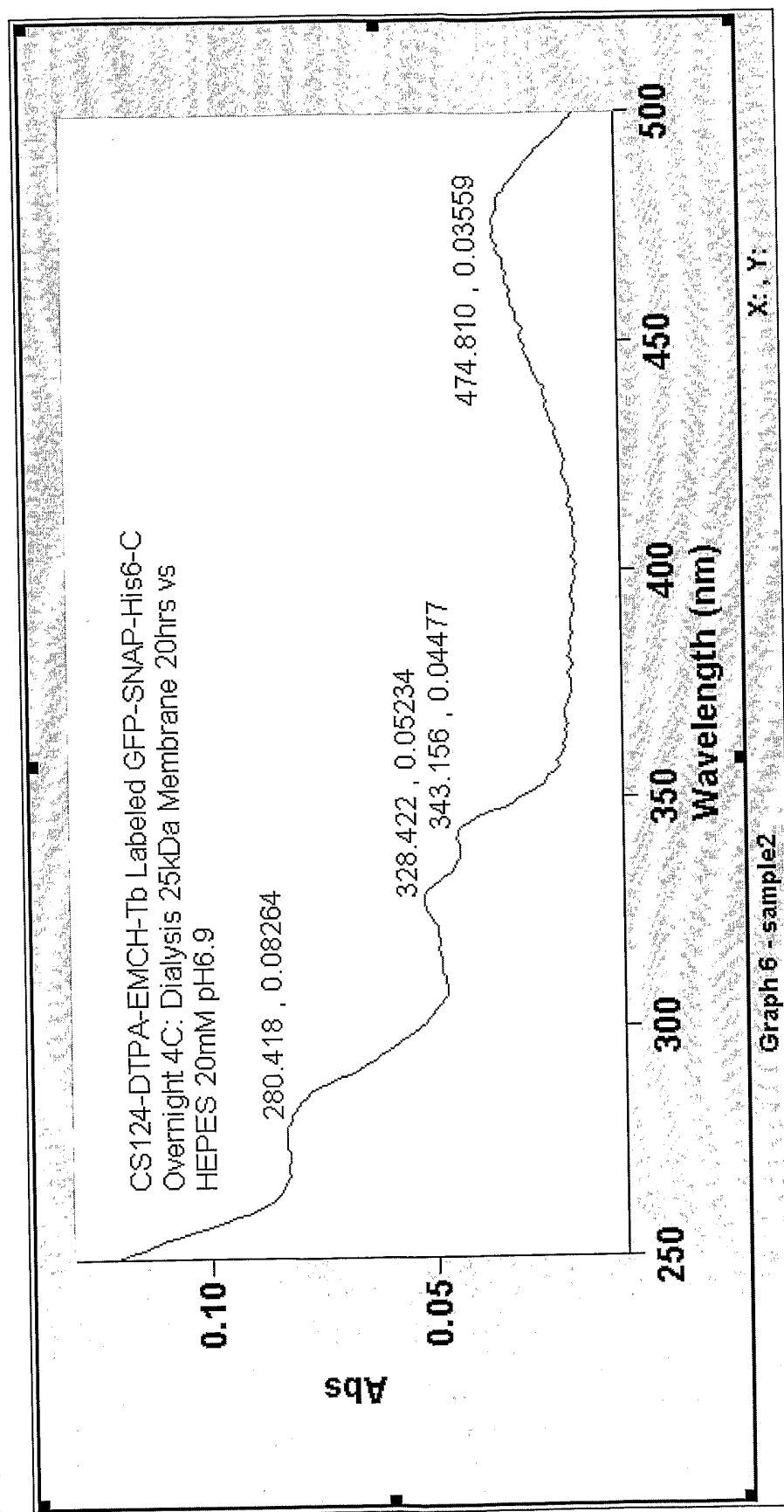


FIG. 9B

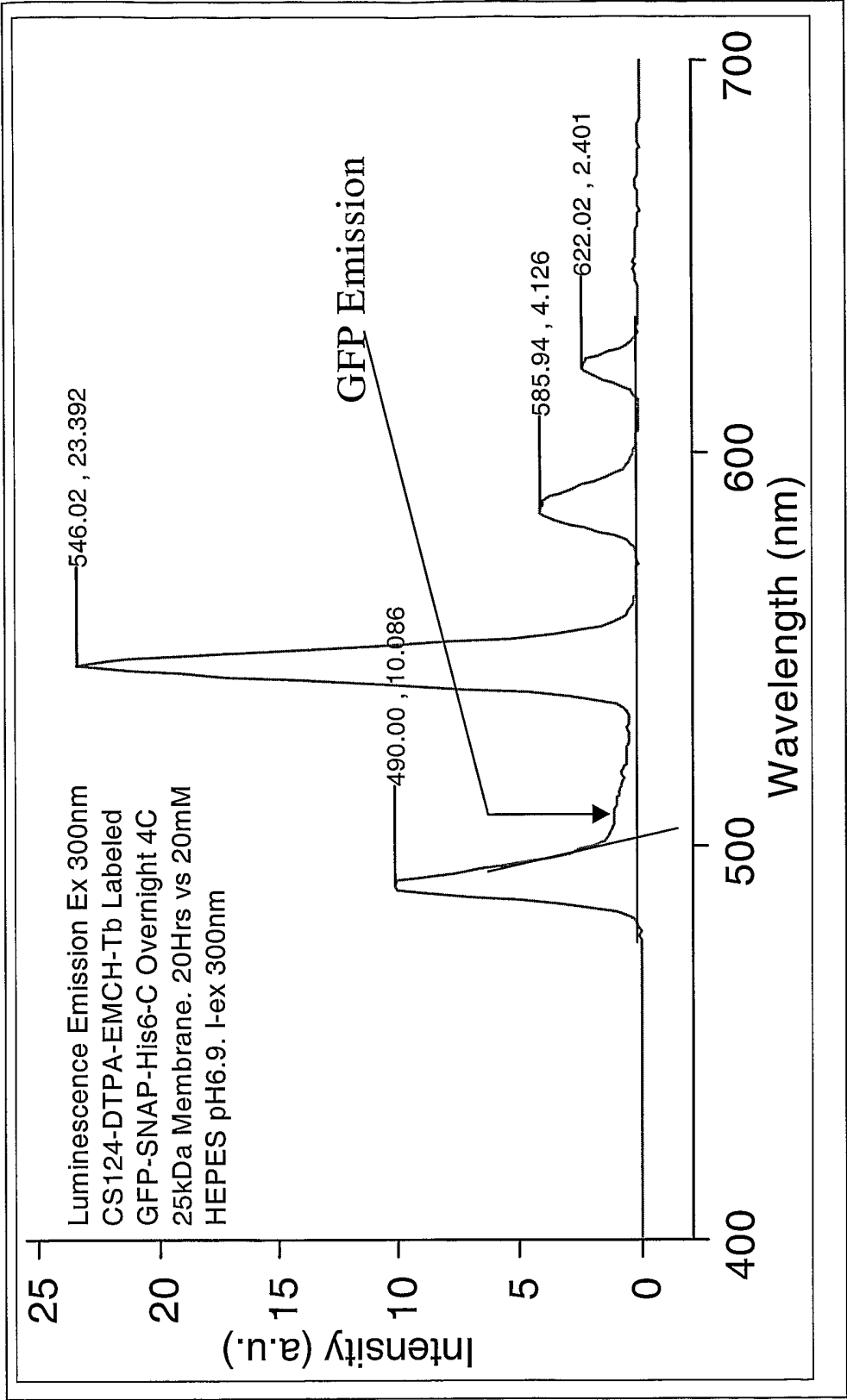


FIG. 10A

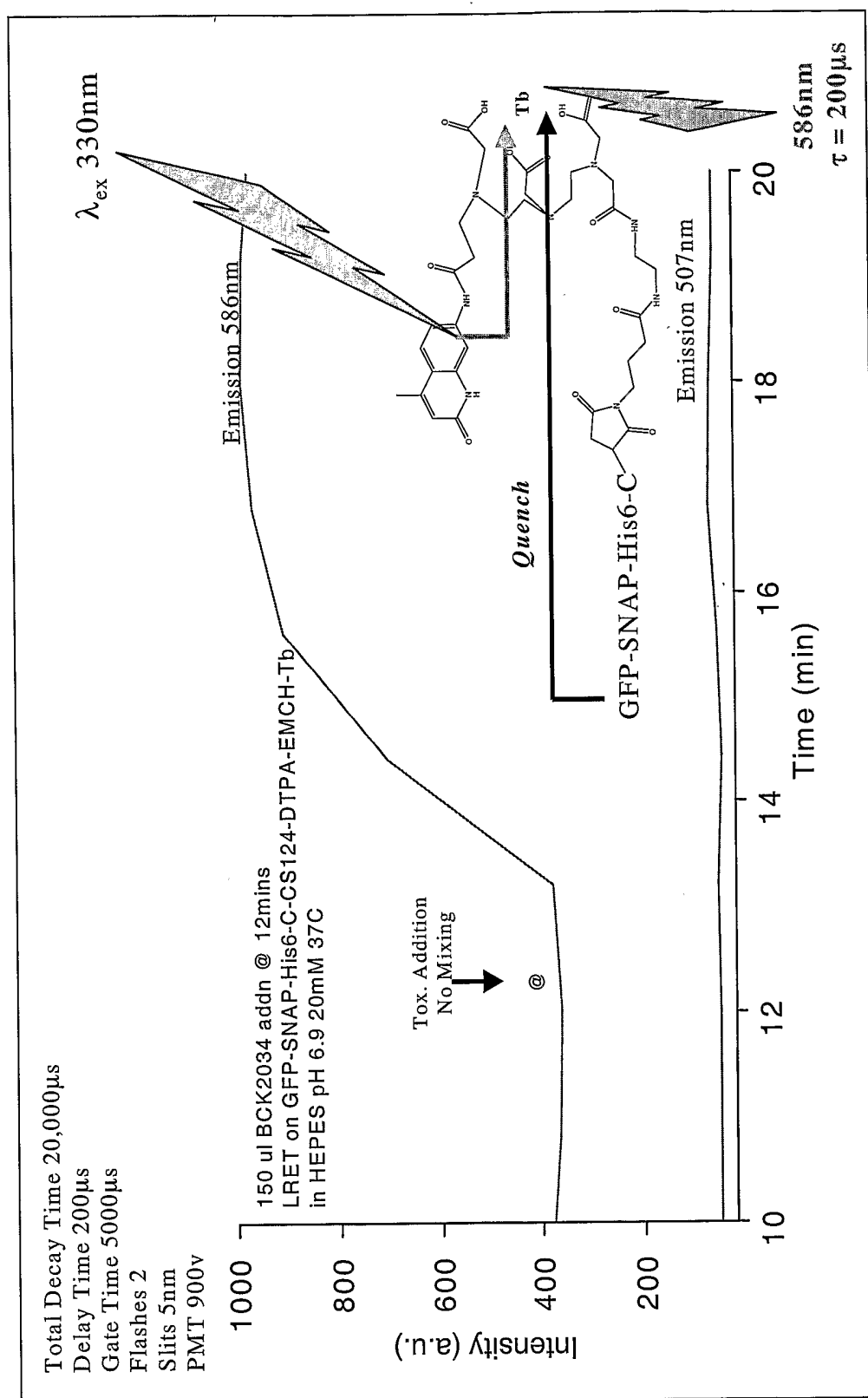
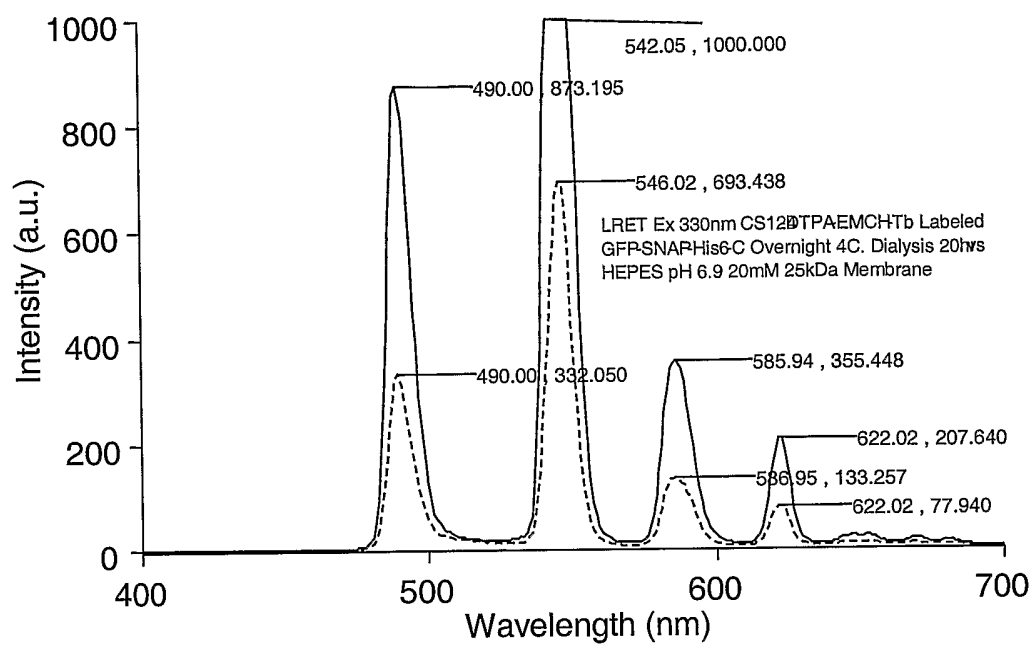


FIG. 10B



SEQUENCE LISTING

<110> Williams, Dudley J.
 Gilmore, Marcella
 Verhagen, Marc
 Steward, Lance
 Aoki, Kei Roger

<120> Lanthanide-Based Substrates and Methods
 For Determining Clostridial Toxin Activity

<130> 66872-043

<160> 101

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 206

<212> PRT

<213> Homo sapiens

<400> 1

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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> 2

<211> 206

<212> PRT

<213> Mus musculus

<400> 2

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> 3

<211> 212

<212> PRT

<213> Drosophila sp.

<400> 3

```

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> 4
 <211> 203
 <212> PRT
 <213> *Carassius auratus*

<400> 4
 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> 5
 <211> 212
 <212> PRT
 <213> *Strongylcentrotas purpuratus*

<400> 5
 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> 6
 <211> 249
 <212> PRT
 <213> Gallus gallus

<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 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 Leu Ile Pro Ile Lys Pro Gly Leu
 180 185 190
 Met Lys Pro Thr Ser Val Gln Gln Arg Cys Ser Ala Val Val Lys Cys
 195 200 205
 Ser Lys Val His Phe Leu Leu Met Leu Ser Gln Arg Ala Val Pro Ser
 210 215 220
 Cys Phe Tyr His Gly Ile Tyr Leu Leu Gly Leu His Thr Cys Thr Tyr
 225 230 235 240
 Gln Pro His Cys Lys Cys Cys Pro Val
 245

<210> 7
 <211> 118
 <212> PRT

<213> Homo sapiens

<400> 7

```

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

```

<210> 8

<211> 116

<212> PRT

<213> Homo sapiens

<400> 8

```

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

```

<210> 9

<211> 116

<212> PRT

<213> Mus musculus

<400> 9

```

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

```

```

      50              55              60
Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65              70              75              80
Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
      85              90              95
Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
      100              105              110
Tyr Phe Ser Thr
      115

```

<210> 10
 <211> 116
 <212> PRT
 <213> Bos taurus

```

<400> 10
Met Ser Ala Thr Ala Ala Thr Ala Pro Pro Ala Ala Pro Ala Gly Glu
 1              5              10              15

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

```

<210> 11
 <211> 114
 <212> PRT
 <213> Xenopus laevis

```

<400> 11
Met Ser Ala Pro Ala Ala Gly Pro Pro Ala Ala Ala Pro Gly Asp Gly
 1              5              10              15
Ala Pro Gln Gly Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu Gln Gln
      20              25              30
Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val Asn Val
      35              40              45
Asp Lys Val Leu Glu Arg Asp Thr Lys Leu Ser Glu Leu Asp Asp Arg
      50              55              60
Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala
65              70              75              80
Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Met Lys Met Met Ile Ile
      85              90              95
Met Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val Tyr Phe
      100              105              110
Ser Thr

```

<210> 12
 <211> 104
 <212> PRT
 <213> Strongylocentrotus purpuratus

<400> 12
 Met Ala Ala Pro Pro Pro Pro Gln Pro Ala Pro Ser Asn Lys Arg Leu
 1 5 10 15
 Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
 20 25 30
 Asn Val Asp Lys Val Leu Glu Arg Asp Gln Ala Leu Ser Val Leu Asp
 35 40 45
 Asp Arg Ala Asp Ala Leu Gln Gln Gly Ala Ser Gln Phe Glu Thr Asn
 50 55 60
 Ala Gly Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Cys Lys Met Met
 65 70 75 80
 Ile Ile Leu Ala Ile Ile Ile Ile Val Ile Leu Ile Ile Ile Ile Val
 85 90 95
 Ala Ile Val Gln Ser Gln Lys Lys
 100

<210> 13
 <211> 288
 <212> PRT
 <213> Homo sapiens

<400> 13
 Met Lys Asp Arg Thr Gln Glu Leu Arg Thr Ala Lys Asp Ser Asp Asp
 1 5 10 15
 Asp Asp Asp Val Ala Val Thr Val Asp Arg Asp Arg Phe Met Asp Glu
 20 25 30
 Phe Phe Glu Gln Val Glu Glu Ile Arg Gly Phe Ile Asp Lys Ile Ala
 35 40 45
 Glu Asn Val Glu Glu Val Lys Arg Lys His Ser Ala Ile Leu Ala Ser
 50 55 60
 Pro Asn Pro Asp Glu Lys Thr Lys Glu Glu Leu Glu Glu Leu Met Ser
 65 70 75 80
 Asp Ile Lys Lys Thr Ala Asn Lys Val Arg Ser Lys Leu Lys Ser Ile
 85 90 95
 Glu Gln Ser Ile Glu Gln Glu Glu Gly Leu Asn Arg Ser Ser Ala Asp
 100 105 110
 Leu Arg Ile Arg Lys Thr Gln His Ser Thr Leu Ser Arg Lys Phe Val
 115 120 125
 Glu Val Met Ser Glu Tyr Asn Ala Thr Gln Ser Asp Tyr Arg Glu Arg
 130 135 140
 Cys Lys Gly Arg Ile Gln Arg Gln Leu Glu Ile Thr Gly Arg Thr Thr
 145 150 155 160
 Thr Ser Glu Glu Leu Glu Asp Met Leu Glu Ser Gly Asn Pro Ala Ile
 165 170 175
 Phe Ala Ser Gly Ile Ile Met Asp Ser Ser Ile Ser Lys Gln Ala Leu
 180 185 190
 Ser Glu Ile Glu Thr Arg His Ser Glu Ile Ile Lys Leu Glu Asn Ser
 195 200 205
 Ile Arg Glu Leu His Asp Met Phe Met Asp Met Ala Met Leu Val Glu
 210 215 220
 Ser Gln Gly Glu Met Ile Asp Arg Ile Glu Tyr Asn Val Glu His Ala
 225 230 235 240

Val Asp Tyr Val Glu Arg Ala Val Ser Asp Thr Lys Lys Ala Val Lys
 245 250 255
 Tyr Gln Ser Lys Ala Arg Arg Lys Lys Ile Met Ile Ile Ile Cys Cys
 260 265 270
 Val Ile Leu Gly Ile Val Ile Ala Ser Thr Val Gly Gly Ile Phe Ala
 275 280 285

<210> 14
 <211> 288
 <212> PRT
 <213> Homo sapiens

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

 195 200 205
 Arg Glu Leu His Asp Met Phe Val Asp Met Ala Met Leu Val Glu Ser
 210 215 220
 Gln Gly Glu Met Ile Asp Arg Ile Glu Tyr Asn Val Glu His Ser Val
 225 230 235 240
 Asp Tyr Val Glu Arg Ala Val Ser Asp Thr Lys Lys Ala Val Lys Tyr
 245 250 255
 Gln Ser Lys Ala Arg Arg Lys Lys Ile Met Ile Ile Ile Cys Cys Val
 260 265 270
 Val Leu Gly Val Val Leu Ala Ser Ser Ile Gly Gly Thr Leu Gly Leu
 275 280 285

<210> 15
 <211> 288
 <212> PRT
 <213> Mus musculus

<400> 15

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Met Lys Asp Arg Thr Gln Glu Leu Arg Thr Ala Lys Asp Ser Asp Asp
 1          5          10          15
Asp Asp Asp Val Thr Val Thr Val Asp Arg Asp Arg Phe Met Asp Glu
          20          25          30
Phe Phe Glu Gln Val Glu Glu Ile Arg Gly Phe Ile Asp Lys Ile Ala
          35          40          45
Glu Asn Val Glu Glu Val Lys Arg Lys His Ser Ala Ile Leu Ala Ser
          50          55          60
Pro Asn Pro Asp Glu Lys Thr Lys Glu Glu Leu Glu Glu Leu Met Ser
65          70          75          80
Asp Ile Lys Lys Thr Ala Asn Lys Val Arg Ser Lys Leu Lys Ser Ile
          85          90          95
Glu Gln Ser Ile Glu Gln Glu Glu Gly Leu Asn Arg Ser Ser Ala Asp
          100          105          110
Leu Arg Ile Arg Lys Thr Gln His Ser Thr Leu Ser Arg Lys Phe Val
          115          120          125
Glu Val Met Ser Glu Tyr Asn Ala Thr Gln Ser Asp Tyr Arg Glu Arg
          130          135          140
Cys Lys Gly Arg Ile Gln Arg Gln Leu Glu Ile Thr Gly Arg Thr Thr
145          150          155          160
Thr Ser Glu Glu Leu Glu Asp Met Leu Glu Ser Gly Asn Pro Ala Ile
          165          170          175
Phe Ala Ser Gly Ile Ile Met Asp Ser Ser Ile Ser Lys Gln Ala Leu
          180          185          190
Ser Glu Ile Glu Thr Arg His Ser Glu Ile Ile Lys Leu Glu Thr Ser
          195          200          205
Ile Arg Glu Leu His Asp Met Phe Met Asp Met Ala Met Leu Val Glu
          210          215          220
Ser Gln Gly Glu Met Ile Asp Arg Ile Glu Tyr Asn Val Glu His Ala
225          230          235          240
Val Asp Tyr Val Glu Arg Ala Val Ser Asp Thr Lys Lys Ala Val Lys
          245          250          255
Tyr Gln Ser Lys Ala Arg Arg Lys Lys Ile Met Ile Ile Ile Cys Cys
          260          265          270
Val Ile Leu Gly Ile Ile Ile Ala Ser Thr Ile Gly Gly Ile Phe Gly
          275          280          285

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<210> 16

<211> 291

<212> PRT

<213> Drosophila sp.

<400> 16

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

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Ser Ala Asp Leu Arg Ile Arg Lys Thr Gln His Ser Thr Leu Ser Arg
    115                120                125
Lys Phe Val Glu Val Met Thr Glu Tyr Asn Arg Thr Gln Thr Asp Tyr
    130                135                140
Arg Glu Arg Cys Lys Gly Arg Ile Gln Arg Gln Leu Glu Ile Thr Gly
    145                150                155                160
Arg Pro Thr Asn Asp Asp Glu Leu Glu Lys Met Leu Glu Glu Gly Asn
    165                170                175
Ser Ser Val Phe Thr Gln Gly Ile Ile Met Glu Thr Gln Gln Ala Lys
    180                185                190
Gln Thr Leu Ala Asp Ile Glu Ala Arg His Gln Asp Ile Met Lys Leu
    195                200                205
Glu Thr Ser Ile Lys Glu Leu His Asp Met Phe Met Asp Met Ala Met
    210                215                220
Leu Val Glu Ser Gln Gly Glu Met Ile Asp Arg Ile Glu Tyr His Val
    225                230                235                240
Glu His Ala Met Asp Tyr Val Gln Thr Ala Thr Gln Asp Thr Lys Lys
    245                250                255
Ala Leu Lys Tyr Gln Ser Lys Ala Arg Arg Lys Lys Ile Met Ile Leu
    260                265                270
Ile Cys Leu Thr Val Leu Gly Ile Leu Ala Ala Ser Tyr Val Ser Ser
    275                280                285
Tyr Phe Met
    290

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<210> 17

<211> 291

<212> PRT

<213> C. elegans

<400> 17

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

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Ser Ile Arg Glu Leu His Asp Met Phe Met Asp Met Ala Met Leu Val
 210 215 220
 Glu Ser Gln Gly Glu Met Val Asp Arg Ile Glu Tyr Asn Val Glu His
 225 230 235 240
 Ala Lys Glu Phe Val Asp Arg Ala Val Ala Asp Thr Lys Lys Ala Val
 245 250 255
 Gln Tyr Gln Ser Lys Ala Arg Arg Lys Lys Ile Cys Ile Leu Val Thr
 260 265 270
 Gly Val Ile Leu Ile Thr Gly Leu Ile Ile Phe Ile Leu Phe Tyr Ala
 275 280 285
 Lys Val Leu
 290

<210> 18

<211> 288

<212> PRT

<213> Strongylocentrotus purpuratus

<400> 18

Met Arg Asp Arg Leu Gly Ser Leu Lys Arg Asn Glu Glu Asp Asp Val
 1 5 10 15
 Gly Pro Glu Val Ala Val Asn Val Glu Ser Glu Lys Phe Met Glu Glu
 20 25 30
 Phe Phe Glu Gln Val Glu Glu Val Arg Asn Asn Ile Asp Lys Ile Ser
 35 40 45
 Lys Asn Val Asp Glu Val Lys Lys Lys His Ser Asp Ile Leu Ser Ala
 50 55 60
 Pro Gln Ala Asp Glu Lys Val Lys Asp Glu Leu Glu Glu Leu Met Ser
 65 70 75 80
 Asp Ile Lys Lys Thr Ala Asn Lys Val Arg Ala Lys Leu Lys Met Met
 85 90 95
 Glu Gln Ser Ile Glu Gln Glu Glu Ser Ala Lys Met Asn Ser Ala Asp
 100 105 110
 Val Arg Ile Arg Lys Thr Gln His Ser Thr Leu Ser Arg Lys Phe Val
 115 120 125
 Glu Val Met Thr Asp Tyr Asn Ser Thr Gln Thr Asp Tyr Arg Glu Arg
 130 135 140
 Cys Lys Gly Arg Ile Gln Arg Gln Leu Glu Ile Thr Gly Lys Ser Thr
 145 150 155 160
 Thr Asp Ala Glu Leu Glu Asp Met Leu Glu Ser Gly Asn Pro Ala Ile
 165 170 175
 Phe Thr Ser Gly Ile Ile Met Asp Thr Gln Gln Ala Lys Gln Thr Leu
 180 185 190
 Arg Asp Ile Glu Ala Arg His Asn Asp Ile Ile Lys Leu Glu Ser Ser
 195 200 205
 Ile Arg Glu Leu His Asp Met Phe Met Asp Met Ala Met Leu Val Glu
 210 215 220
 Ser Gln Gly Glu Met Ile Asp Arg Ile Glu Tyr Asn Val Glu Gln Ser
 225 230 235 240
 Val Asp Tyr Val Glu Thr Ala Lys Met Asp Thr Lys Lys Ala Val Lys
 245 250 255
 Tyr Gln Ser Lys Ala Arg Arg Lys Lys Phe Tyr Ile Ala Ile Cys Cys
 260 265 270
 Gly Val Ala Leu Gly Ile Leu Val Leu Val Leu Ile Ile Val Leu Ala
 275 280 285

<210> 19

<211> 1005

<212> DNA

<213> Artificial Sequence

<220>

<223> Plasmid pQPIGFP-SNAP25

<221> CDS

<222> (1)...(1005)

<400> 19

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	

gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	

180	185	190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt			624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu			
195	200	205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt			672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe			
210	215	220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac ggc			720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Gly			
225	230	235	240
ggg gca gga tcc ggt gcg ggt ggc ggt ggc atc cgg agg gta aca aac			768
Gly Ala Gly Ser Gly Ala Gly Gly Gly Gly Ile Arg Arg Val Thr Asn			
245	250	255	
gat gcc cgg gaa aat gag atg gat gag aac ctg gag cag gtg agc ggc			816
Asp Ala Arg Glu Asn Glu Met Asp Glu Asn Leu Glu Gln Val Ser Gly			
260	265	270	
atc atc gga aac ctc cgc cat atg gct cta gac atg ggc aat gag att			864
Ile Ile Gly Asn Leu Arg His Met Ala Leu Asp Met Gly Asn Glu Ile			
275	280	285	
gac acc cag aat cgc cag atc gac agg atc atg gag aag gct gat tcc			912
Asp Thr Gln Asn Arg Gln Ile Asp Arg Ile Met Glu Lys Ala Asp Ser			
290	295	300	
aac aaa acc aga att gat gaa gcc aac caa cgt gca aca aag atg ctg			960
Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Met Leu			
305	310	315	320
gga agt ggt ggc ggt ggc ggc cat cac cat cac cat cac tgc taa			1005
Gly Ser Gly Gly Gly Gly Gly His His His His His Cys *			
325	330		

<210> 20

<211> 334

<212> PRT

<213> Artificial Sequence

<220>

<223> Plasmid pQBI GFP-SNAP25

<400> 20

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

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

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<210> 21

<211> 5

<212> PRT

<213> Clostridium sp.

<400> 21

Gly Gly Gly Gly Ser

1

5

<210> 22

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 22

Glu Ala Asn Gln Arg Ala Thr Lys

1

5

<210> 23

<211> 8

<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 23
Gly Ala Ser Gln Phe Glu Thr Ser
1 5

<210> 24
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 24
Asp Thr Lys Lys Ala Val Lys Trp
1 5

<210> 25
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 25
Arg Asp Gln Lys Leu Ser Glu Leu
1 5

<210> 26
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 26
Gln Ile Asp Arg Ile Met Glu Lys
1 5

<210> 27
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 27
Glu Arg Asp Gln Lys Leu Ser Glu
1 5

<210> 28
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 28
Glu Thr Ser Ala Ala Lys Leu Lys
1 5

<210> 29
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 29
Gly Ala Ser Gln Phe Glu Thr Ser
1 5

<210> 30
<211> 13
<212> PRT
<213> Homo sapiens

<400> 30
Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Met
1 5 10

<210> 31
<211> 15
<212> PRT
<213> Homo sapiens

<400> 31
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys
1 5 10 15

<210> 32
<211> 16
<212> PRT
<213> Homo sapiens

<400> 32
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Met
1 5 10 15

<210> 33
<211> 17
<212> PRT
<213> Homo sapiens

<400> 33

Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Met
 1 5 10 15
 Leu

<210> 34

<211> 17

<212> PRT

<213> Homo sapiens

<400> 34

Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys
 1 5 10 15
 Met

<210> 35

<211> 18

<212> PRT

<213> Homo sapiens

<400> 35

Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys
 1 5 10 15
 Met Leu

<210> 36

<211> 33

<212> PRT

<213> Mus musculus

<400> 36

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

<210> 37

<211> 32

<212> PRT

<213> Homo sapiens

<400> 37

Gln Asn Pro Gln Ile Lys Arg Ile Thr Asp Lys Ala Asp Thr Asn Arg
 1 5 10 15
 Asp Arg Ile Asp Ile Ala Asn Ala Arg Ala Lys Lys Leu Ile Asp Ser
 20 25 30

<210> 38

<211> 32

<212> PRT

<213> Mus musculus

<400> 38

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

<210> 39

<211> 34

<212> PRT

<213> Gallus gallus

<400> 39

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

Val Lys

<210> 40

<211> 33

<212> PRT

<213> Carassius auratus

<400> 40

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

Gly

<210> 41

<211> 33

<212> PRT

<213> Carassius auratus

<400> 41

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

Gly

<210> 42

<211> 30

<212> PRT

<213> Torpedo sp.

<400> 42

Gln	Asn	Ala	Gln	Val	Asp	Arg	Ile	Val	Val	Lys	Gly	Asp	Met	Asn	Lys
1				5					10					15	
Ala	Arg	Ile	Asp	Glu	Ala	Asn	Lys	His	Ala	Thr	Lys	Met	Leu		
			20					25						30	

<210> 43

<211> 33
 <212> PRT
 <213> Strongylocentrotus purpuratus

<400> 43
 Gln Asn Ser Gln Val Gly Arg Ile Thr Ser Lys Ala Glu Ser Asn Glu
 1 5 10 15
 Gly Arg Ile Asn Ser Ala Asp Lys Arg Ala Lys Asn Ile Leu Arg Asn
 20 25 30
 Lys

<210> 44
 <211> 31
 <212> PRT
 <213> C. elegans

<400> 44
 Gln Asn Arg Gln Leu Asp Arg Ile His Asp Lys Gln Ser Asn Glu Val
 1 5 10 15
 Arg Val Glu Ser Ala Asn Lys Arg Ala Lys Asn Leu Ile Thr Lys
 20 25 30

<210> 45
 <211> 31
 <212> PRT
 <213> Drosophila sp.

<400> 45
 Gln Asn Arg Gln Ile Asp Arg Ile Asn Arg Lys Gly Glu Ser Asn Glu
 1 5 10 15
 Ala Arg Ile Ala Val Ala Asn Gln Arg Ala His Gln Leu Leu Lys
 20 25 30

<210> 46
 <211> 32
 <212> PRT
 <213> Hirudinida sp.

<400> 46
 Gln Asn Arg Gln Val Asp Arg Ile Asn Asn Lys Met Thr Ser Asn Gln
 1 5 10 15
 Leu Arg Ile Ser Asp Ala Asn Lys Arg Ala Ser Lys Leu Leu Lys Glu
 20 25 30

<210> 47

<400> 47
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<210> 48

<400> 48
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<210> 49

<400> 49
000

<210> 50
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 50
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Ala
1 5 10 15
Leu

<210> 51
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<221> MOD_RES
<222> 16
<223> Xaa=Nle

<400> 51
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Lys Xaa
1 5 10 15
Leu

<210> 52
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 52
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr Ala Met
1 5 10 15
Leu

<210> 53
<211> 17
<212> PRT
<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 53

Ser	Asn	Lys	Thr	Arg	Ile	Asp	Glu	Ala	Asn	Gln	Arg	Ala	Ser	Lys	Met
1				5					10					15	

Leu

<210> 54

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<221> MOD_RES

<222> 14

<223> Xaa=Abu

<400> 54

Ser	Asn	Lys	Thr	Arg	Ile	Asp	Glu	Ala	Asn	Gln	Arg	Ala	Xaa	Lys	Met
1				5					10					15	

Leu

<210> 55

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<221> MOD_RES

<222> 13

<223> Xaa=Abu

<400> 55

Ser	Asn	Lys	Thr	Arg	Ile	Asp	Glu	Ala	Asn	Gln	Arg	Xaa	Thr	Lys	Met
1				5					10					15	

Leu

<210> 56

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 56

Ser	Asn	Lys	Thr	Arg	Ile	Asp	Glu	Ala	Asn	Ala	Arg	Ala	Thr	Lys	Met
1				5					10					15	

Leu

<210> 57
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<221> MOD_RES
<222> 11
<223> Xaa=Abu

<400> 57
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Xaa Arg Ala Thr Lys Met
1 5 10 15
Leu

<210> 58
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 58
Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Asn Arg Ala Thr Lys Met
1 5 10 15
Leu

<210> 59
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 59
Ser Asn Lys Thr Arg Ile Asp Glu Ala Ala Gln Arg Ala Thr Lys Met
1 5 10 15
Leu

<210> 60
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic construct

<221> MOD_RES
<222> 9

<223> Xaa=Abu

<400> 60

Ser Asn Lys Thr Arg Ile Asp Glu Xaa Asn Gln Arg Ala Thr Lys Met
 1 5 10 15
 Leu

<210> 61

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 61

Ser Asn Lys Thr Arg Ile Asp Gln Ala Asn Gln Arg Ala Thr Lys Met
 1 5 10 15
 Leu

<210> 62

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 62

Ser Asn Lys Thr Arg Ile Asn Glu Ala Asn Gln Arg Ala Thr Lys Met
 1 5 10 15
 Leu

<210> 63

<211> 40

<212> PRT

<213> Homo sapiens

<400> 63

Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ser Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 64

<211> 40

<212> PRT

<213> Bos taurus

<400> 64

Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15

Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 65
 <211> 40
 <212> PRT
 <213> Rattus sp.

<400> 65
 Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Val Phe Glu Ser Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 66
 <211> 40
 <212> PRT
 <213> Rattus sp.

<400> 66
 Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 67
 <211> 40
 <212> PRT
 <213> Rattus sp.

<400> 67
 Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 68
 <211> 40
 <212> PRT
 <213> Rattus sp.

<400> 68
 Asp Leu Val Ala Gln Arg Gly Glu Arg Leu Glu Leu Leu Ile Asp Lys
 1 5 10 15
 Thr Glu Asn Leu Val Asp Ser Ser Val Thr Phe Lys Thr Thr Ser Arg
 20 25 30
 Asn Leu Ala Arg Ala Met Cys Met
 35 40

<210> 69
 <211> 32
 <212> PRT
 <213> *Gallus gallus*

<400> 69
 Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu
 1 5 10 15
 Gln Ala Gly Ala Ser Val Phe Glu Ser Ser Ala Ala Lys Leu Lys Arg
 20 25 30

<210> 70
 <211> 32
 <212> PRT
 <213> *Gallus gallus*

<400> 70
 Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu
 1 5 10 15
 Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg
 20 25 30

<210> 71
 <211> 40
 <212> PRT
 <213> *Torpedo sp.*

<400> 71
 Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ser Ser Ala Ala
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 72
 <211> 40
 <212> PRT
 <213> *Strongylocentrotus purpuratus*

<400> 72
 Asp Lys Val Leu Asp Arg Asp Gly Ala Leu Ser Val Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Gln Gly Ala Ser Gln Phe Glu Thr Asn Ala Gly
 20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 73
 <211> 40
 <212> PRT
 <213> *Aplysia sp.*

<400> 73
 Glu Lys Val Leu Asp Arg Asp Gln Lys Ile Ser Gln Leu Asp Asp Arg
 1 5 10 15
 Ala Glu Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ala Ser Ala Gly

20 25 30
 Lys Leu Lys Arg Lys Tyr Trp Trp
 35 40

<210> 74
 <211> 40
 <212> PRT
 <213> *Teuthoida* sp.

<400> 74
 Asp Lys Val Leu Glu Arg Asp Ser Lys Ile Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ala Ser Ala Gly
 20 25 30
 Lys Leu Lys Arg Lys Phe Trp Trp
 35 40

<210> 75
 <211> 40
 <212> PRT
 <213> *C. elegans*

<400> 75
 Asn Lys Val Met Glu Arg Asp Val Gln Leu Asn Ser Leu Asp His Arg
 1 5 10 15
 Ala Glu Val Leu Gln Asn Gly Ala Ser Gln Phe Gln Gln Ser Ser Arg
 20 25 30
 Glu Leu Lys Arg Gln Tyr Trp Trp
 35 40

<210> 76
 <211> 40
 <212> PRT
 <213> *Drosophila melanogaster*

<400> 76
 Glu Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Gly Glu Arg
 1 5 10 15
 Ala Asp Gln Leu Glu Gly Gly Ala Ser Gln Ser Glu Gln Gln Ala Gly
 20 25 30
 Lys Leu Lys Arg Lys Gln Trp Trp
 35 40

<210> 77
 <211> 40
 <212> PRT
 <213> *Drosophila melanogaster*

<400> 77
 Glu Lys Val Leu Glu Arg Asp Ser Lys Leu Ser Glu Leu Asp Asp Arg
 1 5 10 15
 Ala Asp Ala Leu Gln Gln Gly Ala Ser Gln Phe Glu Gln Gln Ala Gly
 20 25 30
 Lys Leu Lys Arg Lys Phe Trp Leu
 35 40

<210> 78

<211> 39
 <212> PRT
 <213> Hirudinida sp.

<400> 78
 Asp Lys Val Leu Glu Lys Asp Gln Lys Leu Ala Glu Leu Asp Arg Ala
 1 5 10 15
 Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ala Ser Ala Gly Lys
 20 25 30
 Leu Lys Arg Lys Phe Trp Trp
 35

<210> 79
 <211> 18
 <212> PRT
 <213> Homo sapiens

<400> 79
 Glu Arg Ala Val Ser Asp Thr Lys Lys Ala Val Lys Tyr Gln Ser Lys
 1 5 10 15
 Ala Arg

<210> 80
 <211> 18
 <212> PRT
 <213> Bos taurus

<400> 80
 Glu Arg Ala Val Ser Asp Thr Lys Lys Ala Val Lys Tyr Gln Ser Lys
 1 5 10 15
 Ala Arg

<210> 81
 <211> 18
 <212> PRT
 <213> Rattus rattus

<400> 81
 Glu His Ala Lys Glu Glu Thr Lys Lys Ala Ile Lys Tyr Gln Ser Lys
 1 5 10 15
 Ala Arg

<210> 82
 <211> 18
 <212> PRT
 <213> Rattus rattus

<400> 82
 Glu Lys Ala Arg Asp Glu Thr Arg Lys Ala Met Lys Tyr Gln Gly Gly
 1 5 10 15
 Ala Arg

<210> 83

<211> 18
<212> PRT
<213> Rattus rattus

<400> 83
Glu Arg Gly Gln Glu His Val Lys Ile Ala Leu Glu Asn Gln Lys Lys
1 5 10 15
Ala Arg

<210> 84
<211> 18
<212> PRT
<213> Gallus gallus

<400> 84
Val Pro Glu Val Phe Val Thr Lys Ser Ala Val Met Tyr Gln Cys Lys
1 5 10 15
Ser Arg

<210> 85
<211> 18
<212> PRT
<213> Strongylocentrotus purpuratus

<400> 85
Val Arg Arg Gln Asn Asp Thr Lys Lys Ala Val Lys Tyr Gln Ser Lys
1 5 10 15
Ala Arg

<210> 86
<211> 18
<212> PRT
<213> Aplysia sp.

<400> 86
Glu Thr Ala Lys Met Asp Thr Lys Lys Ala Val Lys Tyr Gln Ser Lys
1 5 10 15
Ala Arg

<210> 87
<211> 18
<212> PRT
<213> Teuthoida sp.

<400> 87
Glu Thr Ala Lys Val Asp Thr Lys Lys Ala Val Lys Tyr Gln Ser Lys
1 5 10 15
Ala Arg

<210> 88
<211> 18
<212> PRT

<213> *Drosophila melanogaster*

<400> 88

Gln Thr Ala Thr Gln Asp Thr Lys Lys Ala Leu Lys Tyr Gln Ser Lys
 1 5 10 15
 Ala Arg

<210> 89

<211> 18

<212> PRT

<213> *Hirudinida* sp.

<400> 89

Glu Thr Ala Ala Ala Asp Thr Lys Lys Ala Met Lys Tyr Gln Ser Ala
 1 5 10 15
 Ala Arg

<210> 90

<211> 116

<212> PRT

<213> *Rattus* sp.

<400> 90

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

<210> 91

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 91

gctagatctc gagttaacca cttcccagca tctttg

36

<210> 92

<211> 24

<212> DNA

<213> Artificial Sequence

<220>
<223> primer

<400> 92
atccggaggg taacaaacga tgcc 24

<210> 93
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 93
cgaattccgc gggccacat gggaggagga ctgaacgaca ttttcgaggc taaaagatc 60

<210> 94
<211> 79
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 94
tcgtttgtta ccctccggat atgatgatga tgatgatgat gatgggatcc atgccactcg 60
atcttttgag cctcgaaga 79

<210> 95
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 95
cgaagatctg gaggactgaa cgacatcttc 30

<210> 96
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 96
gatggtgatg gtgatgacag ccgccaccgc cacc 34

<210> 97
<211> 14
<212> PRT
<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 97

Gly Asp Lys Asn Ala Asp Gly Trp Ile Glu Phe Glu Glu Leu
1 5 10

<210> 98

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 98

Gly Asp Lys Asn Ala Asp Gly Phe Ile Cys Phe Glu Glu Leu
1 5 10

<210> 99

<211> 12

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 99

Asp Lys Asn Ala Asp Gly Cys Ile Glu Phe Glu Glu
1 5 10

<210> 100

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 100

Tyr Ile Asp Thr Asn Asn Asp Gly Trp Tyr Glu Gly Asp Glu Leu Leu
1 5 10 15
Ala

<210> 101

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 101

Thr Glu Arg Arg Gln Gln Leu Asp Lys Asp Gly Asp Gly Thr Ile Asp
1 5 10 15
Glu Arg Glu Ile Lys Ile Trp Phe Gln Asn Lys Arg Ala Lys Ile Lys
20 25 30