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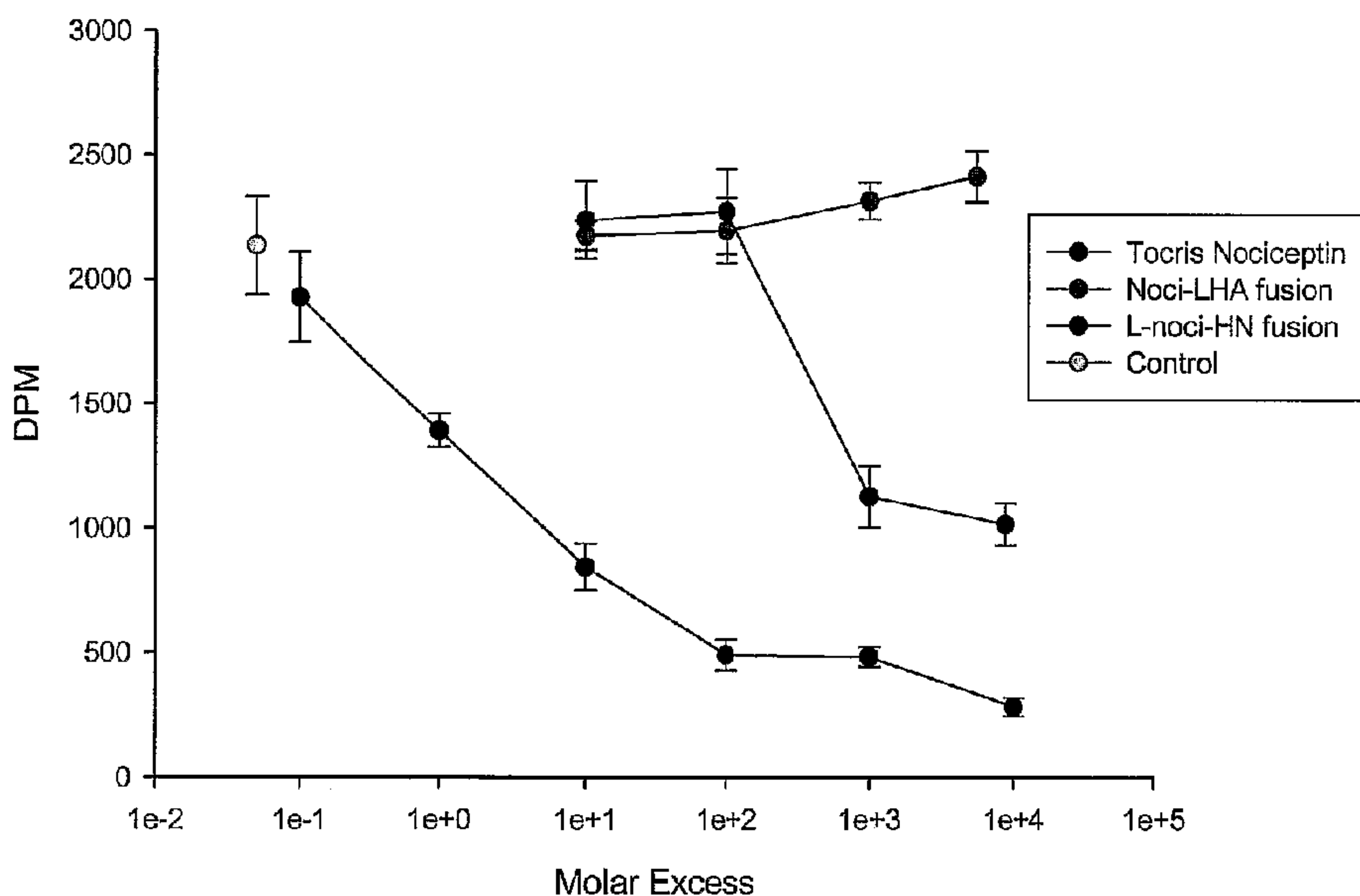
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(54) Titre : CONJUGUES PROTEIQUES NON CYTOTOXIQUES
 (54) Title: NON-CYTOTOXIC PROTEIN CONJUGATES

Competition Assay : Nociceptin-LH_N/A Fusions
 vs 1nM [³H]-Nociceptin on eDRGs (4°C)



(57) **Abrégé/Abstract:**

A non-cytotoxic protein conjugate for inhibition or reduction of exocytic fusion in a nociceptive sensory afferent cell, comprising: (i) a Targeting Moiety (TM), wherein said TM is an agonist of a receptor present on said nociceptive sensory afferent cell, and wherein said receptor undergoes endocytosis to be incorporated into an endosome within the nociceptive sensory afferent cell; (ii) a non-cytotoxic protease or a fragment thereof, wherein the protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of said nociceptive sensory afferent cell; and (iii) a Translocation Domain, wherein the Translocation Domain translocates the protease or protease fragment from within the endosome, across the endosomal membrane, and into the cytosol of the nociceptive sensory afferent cell. Nucleic acid sequences encoding the protein conjugates, methods of preparing same and uses thereof are also described.

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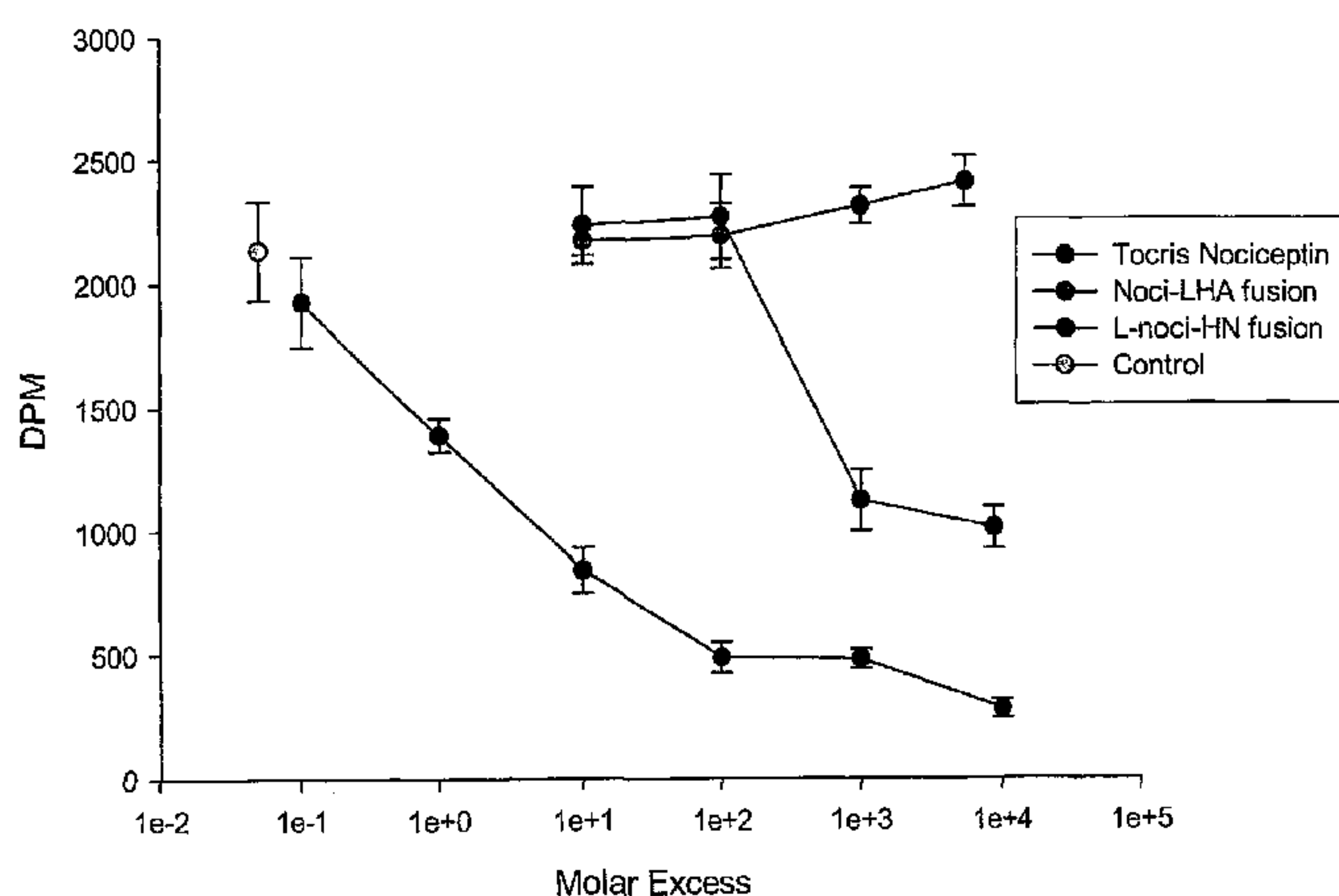
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Non-cytotoxic Protein Conjugates

This invention relates to a non-cytotoxic protein conjugate, and to the use of said
5 conjugate for treating pain.

Toxins may be generally divided into two groups according to the type of effect that they have on a target cell. In more detail, the first group of toxins kill their natural target cells, and are therefore known as cytotoxic toxin molecules. This group of
10 toxins is exemplified *inter alia* by plant toxins such as ricin, and abrin, and by bacterial toxins such as diphtheria toxin, and *Pseudomonas* exotoxin A. Cytotoxic toxins typically kill their target cells by inhibiting the cellular process of protein synthesis.

15 In contrast, the second group of toxins, which are known as non-cytotoxic toxins, do not (as their name confirms) kill their natural target cells. Non-cytotoxic toxins have attracted much less commercial interest than have their cytotoxic counterparts, and exert their effects on a target cell by inhibiting cellular processes other than protein synthesis. As with their cytotoxic counterparts, non-cytotoxic toxins are produced
20 from a variety of sources such as plants, and bacteria. Bacterial non-cytotoxic toxins are now described in more detail.

Clostridial neurotoxins are proteins that typically have a molecular mass of the order of 150 kDa. They are produced by various species of bacteria, especially of the
25 genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*, *C. butyricum* and *C. argentinense*. There are at present eight different classes of the clostridial neurotoxin, namely: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C₁, D, E, F and G, and they all share similar structures and modes of action.

30

Clostridial neurotoxins represent a major group of non-cytotoxic toxin molecules, and

are synthesised by the host bacterium as single polypeptides that are modified post-translationally by a proteolytic cleavage event to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H-chain), which has a molecular mass of approximately 100 kDa, and the light chain (L-chain), which has a molecular mass of approximately 50 kDa.

L-chains possess a protease function (zinc-dependent endopeptidase activity) and exhibit high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytic process. L-chains from different clostridial species or serotypes may hydrolyse different but specific peptide bonds in one of three substrate proteins, namely synaptobrevin, syntaxin or SNAP-25. These substrates are important components of the neurosecretory machinery.

Non-cytotoxic toxins are also produced by other bacteria, such as from the genus *Neisseria*, most importantly from the species *N. gonorrhoeae*. For example, *Neisseria* sp. produces the non-cytotoxic toxin IgA protease (see WO99/58571).

It has been well documented in the art that toxin molecules may be re-targeted to a cell that is not the toxin's natural target cell. When so re-targeted, the modified toxin is capable of binding to a desired target cell and, following subsequent translocation into the cytosol, is capable of exerting its effect on the target cell. Said re-targeting is achieved by replacing the natural Targeting Moiety (TM) of the toxin with a different TM. In this regard, the TM is selected so that it will bind to a desired target cell, and allow subsequent passage of the modified toxin into an endosome within the target cell. The modified toxin also comprises a translocation domain to enable entry of the non-cytotoxic protease into the cell cytosol. The translocation domain can be the natural translocation domain of the toxin or it can be a different translocation domain obtained from a microbial protein with translocation activity.

For example, in the context of non-cytotoxic toxin molecules, it has been well documented that a clostridial neurotoxin may be re-targeted by incorporation of a Targeting Moiety (TM), which is not the natural TM of a clostridial neurotoxin. The

described chemical conjugation and recombinant methodologies are now regarded as conventional, and reference is made to Hermanson, G.T. (1996), Bioconjugate techniques, Academic Press, and to Wong, S.S. (1991), Chemistry of protein conjugation and cross-linking, CRC Press.

5

For example, WO94/21300 describes modified clostridial neurotoxin molecules that are capable of regulating Integral Membrane Protein (IMP) density present at the cell surface of the target cell. The modified neurotoxin molecules are thus capable of controlling cell activity (e.g. glucose uptake) of the target cell. WO96/33273 and
10 WO99/17806 describe modified clostridial neurotoxin molecules that target peripheral sensory afferents. The modified neurotoxin molecules are thus capable of demonstrating an analgesic effect. WO00/10598 describes the preparation of modified clostridial neurotoxin molecules that target mucus hypersecreting cells (or neuronal cells controlling said mucus hypersecreting cells), which modified
15 neurotoxins are capable of inhibiting hypersecretion from said cells. WO01/21213 describes modified clostridial neurotoxin molecules that target a wide range of different types of non-neuronal target cells. The modified molecules are thus capable of preventing secretion from the target cells. Additional publications in the technical field of re-targeted toxin molecules include: WO00/62814; WO00/04926;
20 US5,773,586; WO93/15766; WO00/61192; and WO99/58571.

25

Thus, from the above-described publications, it will be appreciated that the basic concept of re-targeting a non-cytotoxic protease to a desired target cell, by selecting a TM that has a corresponding receptor present on the target cell, has been well documented.

30

However, different receptors present on a target cell of interest demonstrate different binding affinities for different TMs. This may be a particular problem with pain-sensing cells, which possess a wide range of receptor types having different binding
30 affinities for different TMs. Thus, a re-targeted conjugate comprising a particular TM (that binds to a receptor on a pain-sensing cell) may demonstrate a low binding affinity for a pain-sensing target cell, which is undesirable.

There is therefore a need to develop modified non-cytotoxic conjugates that address one or more of the above problems. Of particular interest is the development of an improved conjugate for use in treating pain.

5

The present invention seeks to address one or more of the above problems by using as the conjugate's Targeting Moiety (TM) an "agonist" of a receptor that is present on the pain-sensing target cell of interest. In preferred embodiments, the pain-sensing target cell is a nociceptive sensory afferent, more preferably a primary
10 nociceptive sensory afferent. In particularly preferred embodiments, the TM is an agonist of the opioid-like receptor-1 (ORL₁) receptor.

Accordingly, in a first aspect, the present invention provides a non-cytotoxic conjugate for inhibition or reduction of exocytic fusion in a nociceptive sensory
15 afferent cell, comprising:

(i) a Targeting Moiety (TM),

wherein said TM is an agonist of a receptor present on said
20 nociceptive sensory afferent cell, and wherein said receptor undergoes endocytosis to be incorporated into an endosome within the nociceptive sensory afferent cell;

(ii) a non-cytotoxic protease or a fragment thereof,

25

wherein the protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of said nociceptive sensory afferent cell; and

30 (iii) a Translocation Domain,

wherein the Translocation Domain translocates the protease or

protease fragment from within the endosome, across the endosomal membrane, and into the cytosol of the nociceptive sensory afferent cell.

5 The use of an "agonist", which would normally stimulate a biological process, particularly exocytosis (for example, an increase in cellular secretion, or an up-regulation in membrane protein expression), is an exciting development in the technical field of re-targeted toxins. Furthermore, it is particularly surprising that an agonist may be employed in a therapeutic composition to achieve a reduction or
10 inhibition of a biological process that the agonist would normally stimulate.

The agonist-containing conjugates of the present invention represent a distinct subset of toxin conjugates. In more detail, the conjugates of the present invention comprise TMs that have been selected on the basis of specific agonist properties
15 rather than on the simple basis that they have a corresponding receptor on a pain-sensing target cell of interest.

Conventionally, an agonist has been considered any molecule that can either increase or decrease activities within a cell, namely any molecule that simply causes
20 an alteration of cell activity. For example, the conventional meaning of an agonist would include: a chemical substance capable of combining with a receptor on a cell and initiating a reaction or activity, or a drug that induces an active response by activating receptors, whether the response is an increase or decrease in cellular activity.

25 However, for the purposes of this invention, an agonist is more specifically defined as a molecule that is capable of stimulating the process of exocytic fusion in a pain-sensing target cell, which process is susceptible to inhibition by a protease (or fragment thereof) capable of cleaving a protein of the exocytic fusion apparatus in
30 said target cell.

Accordingly, the particular agonist definition of the present invention would exclude

many molecules that would be conventionally considered as agonists. For example, nerve growth factor (NGF) is an agonist in respect of its ability to promote neuronal differentiation via binding to a TrkA receptor. However, NGF is not an agonist when assessed by the above criteria because it is not a principal inducer of exocytic fusion. In addition, the process that NGF stimulates (i.e. cell differentiation) is not susceptible to inhibition by the protease activity of a non-cytotoxic toxin molecule.

In use, an agonist-containing conjugate of the present invention does not deactivate an agonist receptor on a pain-sensing target cell, but rather the protease activity of the conjugate serves to negate the agonist-mediated response.

Furthermore, once delivered to the cytosol of the pain-sensing target cell, the protease component of a conjugate of the present invention inhibits or blocks the action of all subsequent agonists capable of causing the same effect (i.e. increased exocytic fusion) in the same target cell. This is advantageous and means that the conjugates of the present invention have application in situations where multiple agonists may be responsible for causing the sensation of pain. Thus, when designing a conjugate of the present invention, the TM that is selected for delivery need not necessarily be the principal agonist involved in causing the sensation of pain.

Agonist-mediated delivery according to the present invention provides the following significant advantage over previous non-cytotoxic protease-containing therapeutics: use of an agonist may confer preferential binding and/or internalisation properties on the conjugate. This, in turn, may result in more efficient delivery of the protease component to a pain-sensing target cell.

In addition, use of an agonist as a TM is self-limiting with respect to side-effects. In more detail, binding of an agonist to a pain-sensing target cell increases exocytic fusion, which may exacerbate the sensation of pain. However, the exocytic process that is stimulated by agonist binding is subsequently reduced or inhibited by the protease component of the conjugate.

In preferred embodiments of the invention, the TM is an agonist of the ORL₁ receptor. The ORL₁ receptor is present on pain-sensing cells in the body.

5 The ORL₁ receptor is a member of the G-protein-coupled class of receptors, and has a seven transmembrane domain structure. The properties of the ORL₁ receptor are discussed in detail in Mogil & Pasternak (2001), *Pharmacological Reviews*, Vol. 53, No. 3, pages 381-415.

10 Throughout this specification, reference to the "ORL₁ receptor" embraces all members of the ORL₁ receptor family. Members of the ORL₁ receptor family typically have a seven transmembrane domain structure, and are coupled to G-proteins of the G_i and G_o families. A method for determining the G-protein-stimulating activity of ligands of the ORL₁ receptor is given in Example 17. A method for measuring
15 reduction in cellular cAMP levels following ORL₁ activation is given in Example 16. A further characteristic of members of the ORL₁ receptor family is that they are typically able to bind nociceptin (the natural ligand of ORL₁). As an example, all alternative splice variants of the ORL₁ receptor, are members of the ORL₁ receptor family.

20

The conjugates of the present invention generally demonstrate a reduced binding affinity (in the region of up to 100-fold) for nociceptive sensory afferent target cells when compared with the corresponding 'free' TM. However, despite this observation, the conjugates of the present invention surprisingly demonstrate good
25 efficacy. This can be attributed to two principal features. First, the non-cytotoxic protease component is catalytic – thus, the therapeutic effect of a few such molecules is rapidly amplified. Secondly, the receptors present on the nociceptive sensory afferents need only act as a gateway for entry of the therapeutic, and need not necessarily be stimulated to a level required in order to achieve a ligand-receptor
30 mediated pharmacological response. Accordingly, the conjugates of the present invention may be administered at a dosage that is much lower that would be employed for other types of analgesic molecules such as NSAIDS, morphine, and

gabapentin. The latter molecules are typically administered at high microgram to milligram (even up to hundreds of milligram) quantities, whereas the conjugates of the present invention may be administered at much lower dosages, typically at least 10-fold lower, and more typically at 100-fold lower.

5

In a particularly preferred embodiment of the invention, the TM of the conjugate is nociceptin - the natural ligand for the ORL₁ receptor. Nociceptin targets the ORL₁ receptor with high affinity.

10 Examples of other preferred TMs include:

Code	Sequence	Ref.	SEQ ID No.
Nociceptin 1-17	FGGFTGARKSARKLANQ	[1]	1,2
Nociceptin 1-11	FGGFTGARKSA	[1]	3,4
Nociceptin [Y10]1-11	FGGFTGARKYA	[1]	5,6
Nociceptin [Y11]1-11	FGGFTGARKSY	[1]	7,8
Nociceptin [Y14]1-17	FGGFTGARKSARKYANQ	[1]	9,10
Nociceptin 1-13	FGGFTGARKSARK	[2]	11,12
Nociceptin [R14K15] 1-17 (also known as "variant" nociceptin)	FGGFTGARKSARKRKNQ	[3,4]	13,14
Nociceptin 1-13-NH ₂	FGGFTGARKSARK-NH ₂	[5]	-
Nociceptin Phe (<i>p</i> -NO ₂) 1-17	(<i>p</i> NO ₂)FGGFTGARKSARKLANQ	[5]	-
Lofentanil	Non-peptide agonists	[5]	-

Code	Sequence	Ref.	SEQ ID No.
Etorphine	Non-peptide agonists	[5]	-
Peptide agonist	Peptide agonists from combinatorial library approach	[6]	-

[1] Mogil & Pasternak, 2001, Pharmacol. Rev., 53, 381-415

[2] Maile et al., 2003, Neurosci. Lett., 350, 190-192

[3] Rizzi et al., 2002, J. Pharmacol. Exp. Therap., 300, 57-63

5 [4] Okada et al., 2000, Biochem. Biophys. Res. Commun., 278, 493-498

[5] Zaveri, 2003, Life Sci., 73, 663-678.

[6] Dooley et al., 1997, J Pharmacol Exp Ther. 283(2), 735-41.

The TM preferably comprises a maximum of 50 amino acid residues, more preferably a maximum of 40 amino acid residues, particularly preferably a maximum of 30 amino acid residues, and most preferably a maximum of 20 amino acid residues. For example, nociceptin is a 17 amino acid residue peptide.

The above-identified "variant" TM demonstrates particularly good binding affinity (when compared with natural nociceptin) for nociceptive sensory afferents. Generally speaking, a TM-containing conjugate will demonstrate an approximate 100-fold reduction in binding ability *vis-à-vis* the TM *per se*. The above-mentioned "variant" TM *per se* demonstrates an approximate 3- to 10-fold increase in binding ability for a nociceptive sensory afferent *vis-à-vis* natural nociceptin. Thus, a "variant" TM-containing fusion might be expected to demonstrate an approximate 10-fold reduction in binding ability for a nociceptive sensory afferent *vis-à-vis* 'free' nociceptin. However, the present inventors have demonstrated that conjugates comprising said "variant" TM demonstrate a binding ability that (most surprisingly) closely mirrors that of 'free' nociceptin – see Figure 17.

25

In the context of the present invention, the term agonist of the ORL₁ receptor (such as nociceptin, or any one of the peptides listed in the table above) embraces

molecules having at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% homology with said agonist. The agonist homologues retain the agonist properties of nociceptin at the ORL₁ receptor, which may be tested using the methods provided in Example 10.

5

The invention also encompasses fragments, variants, and derivatives of any one of the TMs described above. These fragments, variants, and derivatives will substantially retain the properties that are ascribed to said TMs.

10 The agonist properties of a TM can be confirmed using the methods described in Example 1. These methods are based on previous experiments (see Inoue *et al.* (1998) Proc. Natl. Acad. Sci., 95, 10949-10953), which confirm that the natural agonist of the ORL₁ receptor, nociceptin, causes the induction of substance P release from nociceptive primary afferent neurons. This is supported by the facts
15 that:

- the nociceptin-induced responses are abolished by specific NK1 receptor (the substance P receptor) antagonists; and
- 20 ➤ pre-treatment of the cells with capsaicin (which depletes substance P from small diameter primary afferent neurons) attenuates the nociceptin-induced responses.

Similarly, Inoue *et al.* confirm that an intraplantar injection of botulinum neurotoxin
25 type A abolishes the nociceptin-induced responses. Since it is known that BoNT inhibits the release of substance P from primary afferent neurons (Welch *et al.*, (2000), Toxicon, 38, 245-258), this confirms the link between nociceptin-ORL₁ interaction and subsequent release of substance P.

30 Thus, a TM can be said to have agonist activity at the ORL₁ receptor if the TM causes an induction in the release of substance P from a nociceptive sensory afferent neuron (see Example 1).

In another embodiment, opioids represent a preferred group of TMs of the present invention. Within this family of peptides is included enkephalins (met and leu), endomorphins 1 and 2, β -endorphin and dynorphin. Opioid peptides are frequently used in the clinic to modify the activity to nociceptors, and other cells involved in the pain response. As exemplified by the three-step World Health Organisation Analgesic Ladder, opioids have entry points into the pharmacological treatment of chronic cancer and non-cancer pain at all three stages, underlining their importance to the treatment of pain. Reference to opioids embraces fragments, variants and derivatives thereof, which retain the ability to bind to nociceptive sensory afferents. The protease of the present invention embraces all naturally-occurring non-cytotoxic proteases that are capable of cleaving one or more proteins of the exocytic fusion apparatus in eukaryotic cells.

The protease of the present invention is preferably a bacterial protease.

More preferably, the bacterial protease is selected from the genera *Clostridium* or *Neisseria* (e.g. a clostridial L-chain, or a neisserial IgA protease preferably from *N. gonorrhoeae*).

The present invention also embraces modified non-cytotoxic proteases, which include amino acid sequences that do not occur in nature and/or synthetic amino acid residues, so long as the modified proteases still demonstrate the above-mentioned protease activity.

The protease of the present invention preferably demonstrates a serine or metalloprotease activity (e.g. endopeptidase activity). The protease is preferably specific for a SNARE protein (e.g. SNAP-25, synaptobrevin/VAMP, or syntaxin).

Particular mention is made to the protease domains of neurotoxins, for example the protease domains of bacterial neurotoxins. Thus, the present invention embraces the use of neurotoxin domains, which occur in nature, as well as recombinantly

prepared versions of said naturally-occurring neurotoxins.

Exemplary neurotoxins are produced by clostridia, and the term clostridial neurotoxin embraces neurotoxins produced by *C. tetani* (TeNT), and by *C. botulinum* (BoNT) serotypes A-G, as well as the closely related BoNT-like neurotoxins produced by *C. baratii* and *C. butyricum*. The above-mentioned abbreviations are used throughout the present specification. For example, the nomenclature BoNT/A denotes the source of neurotoxin as BoNT (serotype A). Corresponding nomenclature applies to other BoNT serotypes.

10

The term L-chain fragment means a component of the L-chain of a neurotoxin, which fragment demonstrates a metalloprotease activity and is capable of proteolytically cleaving a vesicle and/or plasma membrane associated protein involved in cellular exocytosis.

15

A Translocation Domain is a molecule that enables translocation of a protease (or fragment thereof) into a pain-sensing target cell such that a functional expression of protease activity occurs within the cytosol of the target cell. Whether any molecule (e.g. a protein or peptide) possesses the requisite translocation function of the present invention may be confirmed by any one of a number of conventional assays.

20

For example, Shone C. (1987) describes an *in vitro* assay employing liposomes, which are challenged with a test molecule. Presence of the requisite translocation function is confirmed by release from the liposomes of K^+ and/or labelled NAD, which may be readily monitored (see Shone C. (1987) Eur. J. Biochem; vol. 167(1): pp. 175-180).

25

A further example is provided by Blaustein R. (1987), which describes a simple *in vitro* assay employing planar phospholipid bilayer membranes. The membranes are challenged with a test molecule and the requisite translocation function is confirmed by an increase in conductance across said membranes (see Blaustein (1987) FEBS Letts; vol. 226, no. 1: pp. 115-120).

30

Additional methodology to enable assessment of membrane fusion and thus identification of Translocation Domains suitable for use in the present invention are provided by *Methods in Enzymology*, Vols. 220 and 221; Membrane Fusion
 5 Techniques, Parts A and B, Academic Press 1993.

The Translocation Domain is preferably capable of formation of ion-permeable pores in lipid membranes under conditions of low pH. Preferably, it has been found to use only those portions of the protein molecule capable of pore-formation within the
 10 endosomal membrane.

The Translocation Domain may be obtained from a microbial protein source, in particular from a bacterial or viral protein source. Hence, in one embodiment, the Translocation Domain is a translocating domain of an enzyme, such as a bacterial
 15 toxin or viral protein.

It is well documented that certain domains of bacterial toxin molecules are capable of forming such pores. It is also known that certain translocation domains of virally expressed membrane fusion proteins are capable of forming such pores. Such
 20 domains may be employed in the present invention.

The Translocation Domain may be of a clostridial origin, namely the H_N domain (or a functional component thereof). H_N means a portion or fragment of the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. Examples
 25 of suitable clostridial Translocation Domains include:

Botulinum type A neurotoxin	-	amino acid residues (449-871)
Botulinum type B neurotoxin	-	amino acid residues (441-858)
Botulinum type C neurotoxin	-	amino acid residues (442-866)
Botulinum type D neurotoxin	-	amino acid residues (446-862)
Botulinum type E neurotoxin	-	amino acid residues (423-845)

30

Botulinum type F neurotoxin	-	amino acid residues (440-864)
Botulinum type G neurotoxin	-	amino acid residues (442-863)
Tetanus neurotoxin	-	amino acid residues (458-879)

5 For further details on the genetic basis of toxin production in *Clostridium botulinum* and *C. tetani*, we refer to Henderson *et al.* (1997) in *The Clostridia: Molecular Biology and Pathogenesis*, Academic press.

10 The term H_N embraces naturally-occurring neurotoxin H_N portions, and modified H_N portions having amino acid sequences that do not occur in nature and/or synthetic amino acid residues, so long as the modified H_N portions still demonstrate the above-mentioned translocation function.

15 Alternatively, the Translocation Domain may be of a non-clostridial origin (see table below). Examples of non-clostridial Translocation Domain origins include, but are not restricted to, the translocation domain of diphtheria toxin [O'Keefe *et al.*, Proc. Natl. Acad. Sci. USA (1992) 89, 6202-6206; Silverman *et al.*, J. Biol. Chem. (1993) 269, 22524-22532; and London, E. (1992) *Biochem. Biophys. Acta.*, 1112, pp.25-51], the translocation domain of *Pseudomonas* exotoxin type A [Prior *et al.* 20 *Biochemistry* (1992) 31, 3555-3559], the translocation domains of anthrax toxin [Blanke *et al.* Proc. Natl. Acad. Sci. USA (1996) 93, 8437-8442], a variety of fusogenic or hydrophobic peptides of translocating function [Plank *et al.* J. Biol. Chem. (1994) 269, 12918-12924; and Wagner *et al* (1992) *PNAS*, 89, pp.7934-7938], and amphiphilic peptides [Murata *et al* (1992) *Biochem.*, 31, pp.1986-1992].

25 The Translocation Domain may mirror the Translocation Domain present in a naturally-occurring protein, or may include amino acid variations so long as the variations do not destroy the translocating ability of the Translocation Domain.

30 Particular examples of viral Translocation Domains suitable for use in the present invention include certain translocating domains of virally expressed membrane fusion proteins. For example, Wagner *et al.* (1992) and Murata *et al.* (1992) describe the translocation (i.e. membrane fusion and vesiculation) function of a

number of fusogenic and amphiphilic peptides derived from the N-terminal region of influenza virus haemagglutinin. Other virally expressed membrane fusion proteins known to have the desired translocating activity are a translocating domain of a fusogenic peptide of Semliki Forest Virus (SFV); a translocating domain of vesicular stomatitis virus (VSV) glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein. Virally encoded "spike proteins" have particular application in the context of the present invention, for example, the E1 protein of SFV and the G protein of VSV.

Use of the Translocation Domains (listed below) includes use of sequence variants thereof. A variant may comprise one or more conservative nucleic acid substitutions and/or nucleic acid deletions or insertions, with the proviso that the variant possesses the requisite translocating function. A variant may also comprise one or more amino acid substitutions and/or amino acid deletions or insertions, so long as the variant possesses the requisite translocating function.

Translocation Domain source	Amino acid residues	References
Diphtheria toxin	194-380	Silverman <i>et al.</i> , 1994, J. Biol. Chem. 269, 22524-22532 London E. , 1992, Biochem. Biophys. Acta., 1113, 25-51
Domain II of pseudomonas exotoxin	405-613	Prior <i>et al.</i> , 1992, Biochemistry 31, 3555-3559 Kihara & Pastan , 1994, Bioconj Chem. 5, 532-538
Influenza virus haemagglutinin	GLFGAIAGFIENGWE GMIDGWYG, and Variants thereof	Plank <i>et al.</i> , 1994, J. Biol. Chem. 269, 12918-12924 Wagner <i>et al.</i> , 1992, PNAS, 89, 7934-7938 Murata <i>et al.</i> , 1992, Biochemistry 31, 1986-1992

Translocation Domain source	Amino acid residues	References
Semliki Forest virus fusogenic protein	Translocation domain	Kielian <i>et al.</i> , 1996, J Cell Biol. 134(4), 863-872
Vesicular Stomatitis virus glycoprotein G	118-139	Yao <i>et al.</i> , 2003, Virology 310(2), 319-332
SER virus F protein	Translocation domain	Seth <i>et al.</i> , 2003, J Virol 77(11) 6520-6527
Foamy virus envelope glycoprotein	Translocation domain	Picard-Maureau <i>et al.</i> , 2003, J Virol. 77(8), 4722-4730

Once a potential receptor agonist (e.g. an ORL1 agonist) has been identified, one or more of the following optional steps may be carried out:

- 5 (A) confirming that the putative agonist molecule or agonist is capable of being combined with a non-cytotoxic protease (or a fragment thereof) and optionally a Translocation Domain to form a conjugate of the present invention; and/or
- 10 (B) confirming that said putative agonist molecule or agonist binds to the receptor on the pain-sensing target cell, which receptor is susceptible to receptor-mediated endocytosis; and/or
- 15 (C) confirming that said putative agonist molecule or agonist is able to deliver a non-cytotoxic protease (or fragment thereof) into the cytosol of a pain-sensing target cell.

The above steps (A)-(C) may be confirmed by routine tests that would be readily available to a skilled person.

20

For example, step (A) may be performed by a simple chemical conjugation experiment using conventional conjugation reagents and/or linker molecules, followed by native polyacrylamide gel electrophoresis to confirm that a conjugate of ~~the present invention is formed that~~ has the anticipated molecular weight. The
5 conjugate components are typically linked together (optionally via linker molecules) by covalent bonds.

For example, step (B) may be performed by any one of a range of methodologies for assessment of binding of a ligand. Standard text, for example "Receptor-Ligand
10 Interactions. A Practical Approach. Ed. E. C. Hulme, IRL Press, 1992" are available that describe such approaches in detail. In brief, the agonist or putative agonist molecule is labelled (for example, with 125-iodine) and applied to a cell preparation *in vitro* in the presence of an excess of unlabelled agonist. The purpose of the unlabelled material is to saturate any non-specific binding sites. The agonist is
15 incubated with the cell preparation for sufficient time to achieve equilibrium, and the amount of label bound to the cells assessed by measuring cell associated radioactivity, for example by scintillation or gamma counting.

A further example involves gold-labelling of the agonist (or putative agonist),
20 followed by the use of electron microscopy to monitor the cellular transport progress of the labelled agonist [see the basic methodology described by Rabinowitz S. (1992); J. Cell. Biol. 116(1): pp. 95-112; and that described by van Deurs (1986); J. Cell. Biol. 102: pp. 37-47].

For example, step (C) may be performed by contacting the conjugate prepared in step (A) with a suitable target cell and assessing cleavage of the substrate. This is performed by extraction of the SNARE proteins, followed by Western blotting of SDS-PAGE-separated samples. Cleavage of substrate is indicative of delivery of the protease into the target cell. In this regard, cleavage may be monitored by
30 disappearance of substrate and/or appearance of cleavage product. A particularly useful antibody that selectively binds to the cleaved substrate product is described in WO95/33850.

Preparation of a conjugate according to the present invention is now discussed.

5 It is known in the art that the H_C portion of a neurotoxin molecule can be removed from the other portion of the H-chain, known as H_N, such that the H_N fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

10

In another embodiment of the invention, the H_C domain of a neurotoxin is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified neurotoxin is then covalently linked, using linkages which
15 may include one or more spacer regions, to a TM.

20

In another embodiment of the invention, the H-chain of a neurotoxin, in which the H_C domain is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its native binding ability, is combined with the L-chain of a different neurotoxin, or another protease capable of cleaving a protein of the exocytic fusion apparatus (e.g. IgA protease of *N. gonorrhoeae*). This hybrid, modified neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

25

In another embodiment of the invention, the H_N domain of a neurotoxin is combined with the L-chain of a different neurotoxin, or another protease capable of cleaving a protein of the exocytic fusion apparatus (e.g. IgA protease of *N. gonorrhoeae*). This hybrid is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

30

In another embodiment of the invention, the protease (for example the L-chain component of a neurotoxin) is covalently linked, using linkages that may include one

or more spacer regions, to a TM that can also effect the internalisation of the protease into the cytoplasm of the relevant target cell(s).

5 In another embodiment of the invention, the protease (for example the L-chain component of a neurotoxin) is covalently linked, using linkages which may include one or more spacer regions, to a translocation domain to effect transport of the protease fragment into the cytosol.

10 In use, the domains of a conjugate according to the present invention are associated with each other. In one embodiment, two or more of the domains may be joined together either directly (e.g. by a covalent linkage), or via a linker molecule.

15 A variety of different linker/ spacer molecules may be employed in any of the fusion proteins of the present invention. Examples of such spacer molecules include those illustrated in Figures 31 and 32. Particular mention here is made to GS15, GS20, GS25, and Hx27 – see Figures 31 and 32.

20 The present inventors have unexpectedly found that non-cytotoxic protease-TM conjugates (eg. CPNv/A) may demonstrate an improved binding activity for nociceptive sensory afferents when the size of the spacer is selected so that (in use) the TM (preferably the C-terminus thereof) and the translocation domain (preferably the N-terminus thereof) are separated from one another by 40-105 angstroms, preferably by 50-100 angstroms, and more preferably by 50-90 angstroms. In another embodiment, the preferred spacers have an amino acid sequence of 11-29
25 amino acid residues, preferably 15-27 amino acid residues, and more preferably 20-27 amino acid residues. Suitable spacers may be routinely identified and obtained according to Crasto, C.J. and Feng, J.A. (2000) May, 13(5), pp. 309-312 – see also <http://www.fccc.edu/research/labs/feng/linker.html>.

30 Conjugation techniques suitable for use in the present invention have been well documented and are routine for a person skilled in the art.

The methodology involved in coupling two protein molecules (A and B) together is simple, and is achieved through the use of a cross-linking agent (also known as a chemical coupling agent). For example, molecules A and B are separately contacted with a cross-linking agent, which chemically modifies a specific surface group on each of molecules A and B thereby forming derivatised molecules A' and B'. The modified surface group on molecule A' is capable of covalently bonding with the modified surface group on molecule B'. Thus, the coupling reaction is completed by mixing together the two protein molecules A' and B'.

Chemical conjugation is illustrated by reference to the following embodiments, where P = non-cytotoxic protease component, T = translocation component, and TM = targeting moiety.

In one embodiment, a single chain P – T is prepared, which is then conjugated to a TM. In another embodiment, a single chain TM – T (or T – TM) is prepared, which is then conjugated to a P. In a further embodiment, a single chain P – TM (or TM – P) is prepared, which is then conjugated to a T. Another particularly preferred conjugate has the structure P – TM – T (with an optional protease cleavage site between P and TM).

20

Where the T and P components are prepared as a single chain polypeptide, a protease cleavage site is typically included between said components. Any protease cleavage site may be employed in this regard.

In an alternative embodiment, the three components may be simultaneously or sequentially conjugated together. Thus, the conjugation may be a one- or two-step process, and may include one or more different coupling agents.

Chemical coupling agents and cross-linking agents have been commercially available for many years.

30

Example 5 of the present invention describes in detail the use of one such coupling

agent, namely SPDP, to chemically couple two protein molecules (nociceptin, and the LH_N of botulinum neurotoxin). The two molecules are separately contacted with SPDP, and then mixed together to allow covalent conjugation.

- 5 The conjugate described in Example 6 confirms that another coupling agent, PDPH/EDAC, or Traut's reagent, may be employed as an alternative coupling agent to SPDP.

SPDP and Traut's reagent are popular and well-documented coupling agents in the technical field of protein conjugation chemistry and are presented here simply as two
10 examples of a well known class of compounds that may be employed to covalently link together the Targeting Moiety component and the clostridial neurotoxin component of the conjugate of the present invention. Other suitable agents include SMPB, SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexan-1-carboxylate), and
15 LC-SPDP.

In more detail, commercially available members of the well-known coupling agents may be used for conjugation purposes to produce a conjugate of the invention. Details of such agents can be found in the following publications:

20

Hermanson, G.T. (1996), *Bioconjugate techniques*, Academic Press;

Wong, S.S. (1991), *Chemistry of protein conjugation and cross-linking*, CRC Press;

25

Thorpe et al (1987), *Cancer Res*, 1987, 47, 5924-31. This paper describes the use of SMBT (sodium S-4-succinimidylloxycarbonyl-alpha-methyl benzyl thiosulfate) and SMPT (4-succinimidylloxycarbonyl-alpha-methyl-alpha(2-pyridyldithio)toluene);

30

and

Peeters et al (1989), *J Immunol Methods*. 1989, 120, 133-43. This

paper describes the use of 4 coupling reagents, MHS (succinimidyl 6-(N-maleimido)-n-hexanoate), SMCC (succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate), MBS (succinimidyl m-maleimidobenzoate), and SPDP.

5

The conjugates according to the present invention may also be prepared recombinantly, as detailed in Examples 9 to 12.

10

In one embodiment, the preparation of a recombinant conjugate involves arrangement of the coding sequences of a selected TM, a selected non-cytotoxic protease component, and a translocation component (in any order) in a single genetic construct. These coding sequences may be arranged in-frame so that subsequent transcription and translation is continuous through both coding sequences and results in a fusion protein. All constructs would have a 5' ATG codon to encode an N-terminal methionine, and a C-terminal translational stop codon.

15

Thus, the recombinant preparation method results in the generation of a single chain polypeptide. In order to activate this polypeptide, a protease cleavage site is present between the non-cytotoxic protease component and the translocation component. Cleavage of this site generates a di-chain polypeptide in which the protease and translocation domains are linked together by way of a covalent bond, preferably a disulphide bond. In this regard, any protease cleavage site may be employed.

20

In the single polypeptide aspect of the present invention, the TM is preferably either N- or C-terminally located with respect to the fusion protein. In other words, it is preferred that the TM is not located between the P and T components of the single polypeptide fusion protein. In a particularly preferred embodiment, the TM is N-terminally located with respect to the fusion protein.

25

In one embodiment, an L-chain of a clostridial neurotoxin or another protease capable of cleaving a protein of the exocytic fusion apparatus (e.g. an IgA protease), or a fragment/variant thereof, may be expressed recombinantly as a fusion protein

30

with a TM, which TM can also effect the internalisation of the L-chain component into the cytoplasm of the relevant target cell(s) responsible for secretion. Alternatively, the fusion protein may further comprise a Translocation Domain. The expressed fusion protein may include one or more spacer regions.

5

By way of example, the following information is required to produce, recombinantly, an agent of the present invention:

- (I) DNA sequence data relating to a selected TM;
- 10 (II) DNA sequence data relating to the protease component;
- (III) DNA sequence data relating to the translocation domain; and
- (IV) a protocol to permit construction and expression of the construct comprising (I), (II) and (III).

15 All of the above basic information (I)-(IV) are either readily available, or are readily determinable by conventional methods. For example, both WO98/07864 and WO99/17806 exemplify recombinant technology suitable for use in the present application.

20 In addition, methods for the construction and expression of the constructs of the present invention may employ information from the following references and others:

Lorberboum-Galski, H., FitzGerald, D., Chaudhary, V., Adhya, S.,
Pastan, I. (1988), Cytotoxic activity of an interleukin 2-Pseudomonas
25 exotoxin chimeric protein produced in Escherichia coli. Proc.Natl.
Acad. Sci. USA, 85(6):1922-6;

Murphy, J.R. (1988), Diphtheria-related peptide hormone gene
fusions: a molecular genetic approach to chimeric toxin development.
30 Cancer Treat. Res.; 37:123-40;

Williams, D.P., Parker, K., Bacha, P., Bishai, W., Borowski, M.,

Genbauffe, F., Strom, T.B., Murphy, J.R. (1987), Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2-fusion protein. *Protein Eng*;1(6):493-8;

5

Arora, N., Williamson, L.C., Leppla, S.H., Halpern, J.L. (1994), Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells *J. Biol. Chem.*, 269(42):26165-71;

10

Brinkmann, U., Reiter, Y., Jung, S.H., Lee, B., Pastan, I. (1993), A recombinant immunotoxin containing a disulphide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. USA*, 90(16):7538-42; and

15

O'Hare, M.; Brown, A.N., Hussain, K.; Gebhardt, A., Watson, G., Roberts, L.M., Vitetta, E.S., Thorpe, P.E., Lord, J.M. (1990), Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence. *FEBS Lett* Oct 29;273(1-2):200-4.

20

Suitable clostridial neurotoxin sequence information relating to L- and LH_N-chains may be obtained from, for example, Kurazono, H. (1992) *J. Biol. Chem.*, vol. 267, No. 21, pp.14721-14729; and Popoff, M.R., and Marvaud, J.-C. (1999) *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 2nd edition (ed. Alouf, J.E., and Freer, J.H.), Academic Press, pp.174-201.

25

All of the aforementioned publications are hereby incorporated into the present specification by reference thereto.

30

Similarly, suitable TM sequence data are widely available in the art. Alternatively, any necessary sequence data may be obtained by techniques which are well-known to the skilled person.

For example, DNA encoding the TM component may be cloned from a source organism by screening a cDNA library for the correct coding region (for example by using specific oligonucleotides based on the known sequence information to probe the library), isolating the TM DNA, sequencing this DNA for confirmation purposes, and then placing the isolated DNA in an appropriate expression vector for expression in the chosen host.

As an alternative to isolation of the sequence from a library, the available sequence information may be employed to prepare specific primers for use in PCR, whereby the coding sequence is then amplified directly from the source material and, by suitable use of primers, may be cloned directly into an expression vector.

Another alternative method for isolation of the coding sequence is to use the existing sequence information and synthesise a copy, possibly incorporating alterations, using DNA synthesis technology. For example, DNA sequence data may be generated from existing protein and/or RNA sequence information. Using DNA synthesis technology to do this (and the alternative described above) enables the codon bias of the coding sequence to be modified to be optimal for the chosen expression host. This may give rise to superior expression levels of the fusion protein.

Optimisation of the codon bias for the expression host may be applied to the DNA sequences encoding the TM and clostridial components of the construct. Optimisation of the codon bias is possible by application of the protein sequence into freely available DNA/protein database software, e.g. programs available from Genetics Computer Group, Inc.

Having prepared a conjugate of the invention, it is a matter of routine to confirm that the various domains have retained their specified function.

Protease function after conjugation may be tested by using, for example, any one of

the following routine tests:

SNAP-25 (or synaptobrevin, or syntaxin) may be challenged with a conjugate to be tested, and then analysed by SDS-PAGE peptide separation techniques.
5 Subsequent detection of peptides (e.g. by silver staining) having molecular weights corresponding to the cleaved products of SNAP-25 (or other component of the neurosecretory machinery) would confirm the presence of a functional L-chain.

As a further alternative, the conjugate may be tested by assaying for SNAP-25 (or
10 synaptobrevin, or syntaxin) cleavage products via antibody-specific binding (see WO95/33850). In more detail, a specific antibody is employed for detecting cleavage of SNAP-25. Since the antibody recognises cleaved SNAP-25, but not uncleaved SNAP-25, identification of the cleaved product by the antibody confirms the presence of L-chain proteolytic function. By way of exemplification, such a
15 method is described in Examples 2 and 3 of WO96/33273.

Translocation component function after conjugation may be tested using, for example, any one of the following routine tests:

20 Suitable methods are, for example, described by Shone *et al.* (1987) Eur. J. Biochem. 167, pp.175-180; and by Blaustein *et al.* (1987) FEBS 226 (1), pp.115-120.

The Shone *et al.* method employs artificial liposomes loaded with potassium
25 phosphate buffer (pH 7.2) and radiolabelled NAD. Release of K⁺ and NAD from the liposomes correlates with a positive result for channel forming activity and hence translocation activity. In this regard, K⁺ release from liposomes may be measured using an electrode and NAD release calculated by measuring the radioactivity in the supernatant (see page 176, column 1, line 33 - column 2, line 17).

30

The Blaustein *et al.* method employs planar phospholipid bilayer membranes, which are used to test for channel forming activity. In more detail, salt solutions on either

side of the membrane are buffered at a different pH - on the cis side, pH 4.7 or 5.5 and on the trans side, pH 7.4. The "conjugate" to be tested is added to the cis side of the membrane and electrical measurements are made under voltage clamp conditions, in order to monitor the flow of current across the membrane (see paragraph 2.2, pages 116-118). The presence of an active translocation function is confirmed by a steady rate of channel turn-on (i.e. a positive result for channel formation) -see paragraph 3, page 118.

Targeting Moiety (TM) function after conjugation may be tested by assaying for the agonist function inherent to the TM. Suitable methods include those described in Example 1.

The ability of the conjugate of the invention to inhibit substance P release from nociceptive afferent cells can be assessed using the methods described in Example 15.

In Example 15, a nociceptin-LHN/A conjugate according to the first aspect of the invention is assessed for its ability to inhibit the release of substance P from primary nociceptive sensory afferent neurons. As can be seen from Table 1, incubation of the conjugate with cultures of nociceptive afferent neurons results in a significant inhibition of release of substance P (when compared to incubation of the cells with LHN/A alone). The experiment therefore confirms that the conjugate is inhibiting substance P release from these cells.

In use of the present invention, a pain-sensing target cell is selected in which it is desired to reduce or inhibit the process of exocytic fusion, which exocytic process contributes to the symptoms associated with the sensation of pain. For example, the target cell in question may demonstrate an undesirable phenotype (e.g. an undesirable secretion, or the expression of an undesirable concentration of membrane receptor, transporter or membrane channel), which contributes to the symptoms associated with pain. Alternatively, a target cell may be selected in which the process of exocytic fusion contributes to the sensation of pain.

In preferred embodiments of the invention, the target cell is a nociceptive sensory afferent cell, preferably a primary nociceptive afferent cell (e.g. an A-fibre such as an A δ -fibre or a C-fibre). Thus, the conjugates of the present invention are capable of inhibiting neurotransmitter or neuromodulator (e.g. glutamate, substance P, calcitonin-gene related peptide (CGRP), and/or neuropeptide Y) release from discrete populations of nociceptive sensory afferent neurons. In use, the conjugates reduce or prevent the transmission of sensory afferent signals (e.g. neurotransmitters or neuromodulators) from peripheral to central pain fibres, and therefore have application as therapeutic molecules for the treatment of pain, in particular chronic pain.

It is routine to confirm that a TM binds to a nociceptive sensory afferent. For example, a simple radioactive displacement experiment may be employed in which tissue or cells representative of the nociceptive sensory afferent (for example DRGs) are exposed to labelled (e.g. tritiated) ligand in the presence of an excess of unlabelled ligand. In such an experiment, the relative proportions of non-specific and specific binding may be assessed, thereby allowing confirmation that the ligand binds to the nociceptive sensory afferent target cell. Optionally, the assay may include one or more binding antagonists, and the assay may further comprise observing a loss of ligand binding. Examples of this type of experiment can be found in Hulme, E.C. (1990), Receptor-binding studies, a brief outline, pp 303-311, in Receptor biochemistry, A Practical Approach, Ed. E.C. Hulme, Oxford University Press.

According to a second aspect, the present invention provides a non-cytotoxic conjugate for inhibition or reduction of exocytotic fusion in a nociceptive sensory afferent cell, comprising:

(i) a Targeting Moiety (TM),

wherein said TM is an agonist of a receptor that is present on

said nociceptive sensory afferent cell, and wherein said receptor undergoes endocytosis to be incorporated into an endosome within the nociceptive sensory afferent cell;

- 5 (ii) a DNA sequence encoding a non-cytotoxic protease or a fragment thereof,

wherein the DNA sequence is expressible in the nociceptive sensory afferent cell and when so expressed provides a protease or protease fragment capable of cleaving a protein of the exocytic fusion apparatus of said nociceptive sensory afferent cell; and

10

- (iii) a Translocation Domain,
- 15

wherein the Translocation Domain translocates the DNA sequence encoding the protease or protease fragment from within the endosome, across the endosomal membrane, and into the nociceptive sensory afferent cell.

20

In a preferred embodiment, the receptor is an ORL₁ receptor.

DNA encoding a protein of interest can be transfected into eukaryotic cells through receptor-mediated endocytosis of a protein-DNA conjugate, as confirmed by Cotton *et al.* (Cotton, M., Wagner, E. and Birnstiel, L. (1993) Receptor-mediated transport of DNA into eukaryotic cells. *Methods in Enzymol.* 217, 619-645). Several methods exist for condensing DNA to a suitable size using polycationic ligands. These include: polylysine, various cationic peptides and cationic liposomes. Of these, polylysine was used in the present study because of its successfully reported use in receptor-mediated transfection studies (Cotton *et al.*, 1993).

25

30

The DNA sequence encoding the non-cytotoxic protease component may be

expressed under the control of an operably linked promoter present as part of the agent (e.g. as part of the protease DNA sequence upstream of the coding region). Alternatively, expression of the protease component in the target cell may rely on a promoter present in the target cell.

5

The DNA sequence encoding the protease component may integrate into a DNA sequence of the target cell. One or more integration site(s) may be provided as part of the conjugate (e.g. as part of the protease DNA sequence).

10 The TM, Translocation Domain and protease components of this second aspect of the invention are as defined for the first aspect of the invention. Examples 13 and 14 describe the preparation of conjugates according to the second aspect of the invention.

15 According to a third aspect, the present invention provides a pharmaceutical composition comprising a conjugate according to the first and/or second aspect of the present invention.

20 The pharmaceutical composition may further comprise a pharmaceutically-acceptable carrier, and/or a suitable diluent and/or excipient, although the exact form of the composition may be tailored to the mode of administration. Administration is preferably to a mammal, more preferably to a human.

25 The components of the composition may, for example, be employed in the form of an aerosol or nebulisable solution for inhalation or a sterile solution for parenteral administration, intra-articular administration or intra-cranial administration.

30 The composition may also be administered by i.v. injection, which includes the use of pump systems. Spinal injection (e.g. epidural or intrathecal) or indwelling pumps may also be used.

The dosage ranges for administration of the components of the present invention are

those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the components, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician.

Suitable daily dosages (for each component) are in the range 0.0001-1 mg/kg, preferably 0.0001-0.5 mg/kg, more preferably 0.002-0.5 mg/kg, and particularly preferably 0.004-0.5 mg/kg. The unit dosage can vary from less than 1 microgram to 30 mg, but typically will be in the region of 0.01 to 1 mg per dose, which may be administered daily or preferably less frequently, such as weekly or six monthly.

A particularly preferred dosing regimen is based on 2.5 ng of fusion protein (e.g. CPNv/A) as the 1X dose. In this regard, preferred dosages are in the range 1X–100X (i.e. 2.5-250 ng). This dosage range is significantly lower (i.e. at least 10-fold, typically 100-fold lower) than would be employed with other types of analgesic molecules such as NSAIDs, morphine, and gabapentin. Moreover, the above-mentioned difference is considerably magnified when the same comparison is made on a molar basis – this is because the fusion proteins of the present invention have a considerably greater Mw than do conventional 'small' molecule therapeutics.

Wide variations in the required dosage, however, are to be expected depending on the precise nature of the components, and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection.

Variations in these dosage levels can be adjusted using standard empirical routines for optimisation, as is well understood in the art.

Compositions suitable for injection may be in the form of solutions, suspensions or emulsions, or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

Fluid unit dosage forms are typically prepared utilising a pyrogen-free sterile vehicle.

~~The active ingredients, depending on the vehicle and concentration used, can be~~
5 either dissolved or suspended in the vehicle.

Solutions may be used for all forms of parenteral administration, and are particularly used for intravenous injection. In preparing solutions the components can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of
10 sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving.

15 Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents and/or local anaesthetic agents may be dissolved in the vehicle.

Dry powders which are dissolved or suspended in a suitable vehicle prior to use may
20 be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area.

Alternatively the components of the composition may be dissolved in an aqueous vehicle, the solution is sterilized by filtration and distributed into suitable containers
25 using aseptic technique in a sterile area. The product is then freeze-dried and the containers are sealed aseptically.

Parenteral suspensions, suitable for intramuscular, subcutaneous or intradermal injection, are prepared in substantially the same manner, except that the sterile
30 components are suspended in the sterile vehicle, instead of being dissolved and sterilisation cannot be accomplished by filtration. The components may be isolated in a sterile state or alternatively it may be sterilised after isolation, e.g. by gamma

irradiation.

Advantageously, a suspending agent for example polyvinylpyrrolidone is included in the composition(s) to facilitate uniform distribution of the components.

5

Compositions suitable for administration via the respiratory tract include aerosols, nebulisable solutions or microfine powders for insufflation. In the latter case, particle size of less than 50 microns, especially less than 10 microns, is preferred. Such compositions may be made up in a conventional manner and employed in conjunction with conventional administration devices.

10

The compositions described in this invention can be used in vivo, either directly or as a pharmaceutically acceptable salt, for the treatment of conditions involving exocytosis (for example secretion, or the delivery of proteins such as receptors, transporters, and membrane channels to the plasma membrane of a cell).

15

According to a fourth aspect, the present invention provides a DNA construct that encodes a conjugate according to the first or second aspects of the invention.

By expressing the construct in a host cell, conjugates of the invention may be prepared.

20

According to a fifth aspect, the present invention provides a method of treatment of pain by administration to a patient of a conjugate, composition, or construct according to the first to fourth aspects of the invention, or any combination thereof.

25

In a preferred embodiment, the invention provides a method of treating chronic pain.

According to a sixth aspect, the present invention provides for the use of a conjugate, composition or construct according to the first to fourth aspects of the invention, for the manufacture of a medicament for treating pain, preferably chronic pain.

30

Definitions Section

Exocytic fusion is a process by which intracellular molecules are transported from
5 the cytosol of a pain-sensing target cell to the plasma (i.e. cell) membrane thereof.
Thereafter, the intracellular molecules may become displayed on the outer surface of
the plasma membrane, or may be secreted into the extracellular environment.

10 In a healthy individual, the rate of exocytic fusion is carefully regulated and allows
control of the transport of molecules between the cytosol and the plasma membrane
of a pain-sensing cell. For example, regulation of the exocytic cycle allows control of
the density of receptors, transporters, or membrane channels present at the cell's
surface, and/or allows control of the secretion rate of intracellular components (e.g.
neurotransmitters) from the cytosol of the cell.

15 However, in an unhealthy individual, the regulation of exocytic fusion may be
modified. For example, exocytic fusion may cause affected pain-sensing cells to
enter a state of hypersecretion. Alternatively, exocytic fusion may result in the
display of an increased concentration of receptors, transporters, or membrane
20 channels present on the surface of the pain-sensing, which may expose the cell to
undesirable external stimuli. Thus, the process of exocytic fusion may contribute to
the progression and/or severity of pain, and therefore provides a target for
therapeutic intervention.

25 It should also be appreciated that otherwise normal rates of cellular exocytic fusion
may contribute to the progression and severity of pain in compromised patients.
Thus, by targeting exocytic fusion in accordance with the present invention, it is also
possible to provide therapy in such patients

30 Targeting Moiety (TM) means any chemical structure associated with a conjugate
that functionally interacts with a receptor, e.g. an ORL₁ receptor, to cause a physical
association between the conjugate and the surface of a pain-sensing target cell.

The term TM embraces any molecule (i.e. a naturally occurring molecule, or a chemically/physically modified variant thereof) that is capable of binding to a receptor on the target cell, which receptor is capable of internalisation (e.g. endosome-formation) – also-referred-to as-receptor-mediated-endocytosis. The TM
5 may possess an endosomal membrane translocation domain, in which case separate TM and Translocation Domain components need not be present in an agent of the present invention.

The term “fragment” means a peptide having at least thirty-five, preferably at least
10 twenty-five, more preferably at least fifteen, and most preferably at least ten amino acid residues of the TM in question. In one embodiment, the first amino acid residue of the fragment is the N-terminal amino acid residue of the TM from which the fragment has been derived.

15 An example of a “variant” is a peptide or peptide fragment of a TM that contains one or more analogues of an amino acid (e.g. an unnatural amino acid), or a substituted linkage.

A “derivative” comprises the TM in question, and a further peptide sequence. The
20 further peptide sequence should preferably not interfere with the basic folding and thus conformational structure of the TM. Two or more peptides (or fragments, or variants) may be joined together to form a derivative. Alternatively, a peptide (or fragment, or variant) may be joined to an unrelated molecule (e.g. a second, unrelated peptide). Derivatives may be chemically synthesized, but will be typically
25 prepared by recombinant nucleic acid methods. Additional components such as lipid, and/or polysaccharide, and/or polyketide components may be included.

The term non-cytotoxic means that the protease molecule in question does not kill the pain-sensing target cell to which it has been re-targeted.

30

The “protease cleavage site” of the present invention allows cleavage (preferably controlled cleavage) of the conjugate at a position between the non-cytotoxic

protease component and the TM component. In one embodiment, the conjugate may include more than one proteolytic cleavage site. However, where two or more such sites exist, they are different, thereby substantially preventing the occurrence of multiple cleavage events in the presence of a single protease. In another embodiment, it is preferred that the conjugate has a single protease cleavage site. The protease cleavage sequence(s) may be introduced (and/or any inherent cleavage sequence removed) at the DNA level by conventional means, such as by site-directed mutagenesis. Screening to confirm the presence of cleavage sequences may be performed manually or with the assistance of computer software (e.g. the MapDraw program by DNASTAR, Inc.).

Whilst any protease cleavage site may be employed, the following are preferred:

Enterokinase	(DDDDK↓)
Factor Xa	(IEGR↓ / IDGR↓)
TEV(Tobacco Etch virus)	(ENLYFQ↓G)
Thrombin	(LVPR↓GS)
PreScission	(LEVLFQ↓GP).

Also embraced by the term protease cleavage site is an intein, which is a self-cleaving sequence. The self-splicing reaction is controllable, for example by varying the concentration of reducing agent present.

The present invention is now described by reference to the following Examples and Figures, without intended limitation thereto.

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- Figure 1 Expression and purification of *recLH_N/B* fusion protein
- Figure 2 Expression and purification of *LH_N/C* fusion protein
- Figure 3 Expression and purification of *N[1-17]-LH_N/A* fusion protein
- Figure 4 Purification of a *LC/A-nociceptin-H_N/A* fusion protein

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- Figure 6 Purification of a LC/C-nociceptin-H_N/C fusion protein
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- Figure 9 *In vitro* catalytic activity of a LC/A-nociceptin-H_N/A fusion protein
- Figure 10 Purification of a LC/A-nociceptin variant-H_N/A fusion protein
- Figure 11 Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion
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- Figure 29 *In vitro* SNAP-25 cleavage in a DRG cell model
- Figure 30 Expressed / purified CPNv-A-FXa-HT (removable his-tag)
- Figure 31 *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable
spacer length, as assessed by ligand competition assay
- 5 Figure 32 *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable
spacer length, as assessed by *in vitro* SNAP-25 cleavage

The Figures are now described in more detail.

10 **Figure 1 - Expression and purification of *rec*LH_N/B fusion protein**

SDS-PAGE analysis of expression and purification of *rec*LH_N/B from *E. coli*. In Figure 1, *rec*LH_N/B is purified from cell paste using a three column strategy as described in Example 3. Protein samples are separated by SDS-PAGE and
15 visualised by staining with simplyblue safestain coomassie reagent. Crude, soluble MBP-LH_N/B fusion protein contained within the clarified extract (lane 2) is loaded onto Q-Sepharose FF anion-exchange resin. Lane 3 represents recombinant MBP-LH_N/B fusion eluted from column at 150-200 mM salt. This sample is treated with factor Xa protease to remove MBP affinity tag (lane 4), and cleaved mixture diluted
20 to lower salt concentration prior to loading onto a Q-Sepharose FF anion-exchange column. Material eluted between 120-170 mM salt was rich in LH_N/B (lane 5). Protein in lanes 6 and 8 represents LH_N/B harvested after treatment with enterokinase and final purification using Benzamidine Sepharose, under non-reducing and reducing conditions respectively. Lanes 1 and 7 represent molecular
25 mass markers [Mark 12 (Invitrogen)].

Figure 2 - Expression and purification of LH_N/C fusion protein

SDS-PAGE analysis of expression and purification of LH_N/C from *E. coli*. In Figure
30 2, *rec*LH_N/C is purified from *E. coli* cell paste using a two-step strategy described in Example 4. Protein samples are separated by SDS-PAGE and visualised by staining with coomassie blue. Clarified Crude cell lysate (lane 2) is loaded onto Q-

Sepharose FF anion-exchange resin. Fusion protein, MBP-LH_N/C is eluted with 0.1 M NaCl (lane 3). Eluted material incubated at 22°C for 16 h with factor Xa protease (New England Biolabs) to cleave fusion tag MBP and nick *rec*LH_N/C at the linker site. The protein of interest is further-purified from cleaved fusion products (lane 4) using Q-Sepharose FF. Lanes 5 and 7 show purified *rec*LH_N/C under non-reducing conditions and reduced with 10 mM DTT respectively, to illustrate disulphide bonding at the linker region between LC and H_N domains after nicking with factor Xa. Lanes 1 and 6 represent molecular mass markers (shown in KDa); Mark 12 (Invitrogen).

10 **Figure 3 - Expression and purification of N[1-17]-LH_N/A fusion protein**

SDS-PAGE analysis of expression and purification of N[1-17]-LH_N/A from *E. coli*. In Figure 3, N[1-17]-LH_N/A is purified from *E. coli* BL21 cell paste using the methodology outlined in Example 9. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

25 **Figure 4 - Purification of a LC/A-nociceptin-H_N/A fusion protein**

Using the methodology outlined in Example 26, a LC/A-nociceptin-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting

(Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

5 **Figure 5 - Purification of a nociceptin-LC/A-H_N/A fusion protein**

Using the methodology outlined in Example 26, a nociceptin-LC/A-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 6 - Purification of a LC/C-nociceptin-H_N/C fusion protein

20 Using the methodology outlined in Example 26, an LC/C-nociceptin-H_N/C fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE (Panel A) and Western blotting (Panel B). Anti-nociceptin antisera (obtained from Abcam) were used as the primary antibody for Western blotting. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

Figure 7 - Purification of a LC/A-met enkephalin-H_N/A fusion protein

Using the methodology outlined in Example 26, an LC/A-met enkephalin-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+]
5
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Figure 8 - Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion protein and a nociceptin-LC/A-H_N/A fusion protein

The ability of nociceptin fusions to bind to the ORL₁ receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin-H_N/A fusion is far superior to the nociceptin-LC/A-H_N/A fusion at interacting with the ORL₁ receptor.
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Figure 9 - *In vitro* catalytic activity of a LC/A-nociceptin-H_N/A fusion protein

The *in vitro* endopeptidase activity of the purified LC/A-nociceptin-H_N/A fusion protein was determined essentially as described in Chaddock *et al* 2002, Prot. Express Purif. 25, 219-228. Briefly, SNAP-25 peptide immobilised to an ELISA plate was exposed to varying concentrations of fusion protein for 1 hour at 37°C.
30

Following a series of washes, the amount of cleaved SNAP-25 peptide was quantified by reactivity with a specific antisera.

Figure 10 - Purification of a LC/A-nociceptin variant-H_N/A fusion protein

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Using the methodology outlined in Example 26, an LC/A-nociceptin variant-H_N/A fusion protein was purified from *E. coli* BL21 cells. Briefly, the soluble products obtained following cell disruption were applied to a nickel-charged affinity capture column. Bound proteins were eluted with 100 mM imidazole, treated with Factor Xa
10 to activate the fusion protein and remove the maltose-binding protein (MBP) tag, then re-applied to a second nickel-charged affinity capture column. Samples from the purification procedure were assessed by SDS-PAGE. The final purified material in the absence and presence of reducing agent is identified in the lanes marked [-] and [+] respectively.

15

Figure 11 - Comparison of binding efficacy of a LC/A-nociceptin-H_N/A fusion protein and a LC/A-nociceptin variant-H_N/A fusion protein

The ability of nociceptin fusions to bind to the ORL₁ receptor was assessed using a
20 simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin variant-H_N/A
25 fusion (CPNv-LHA) is superior to the LC/A-nociceptin variant-H_N/A fusion (CPN-LHA) at interacting with the ORL₁ receptor.

Figure 12 - Expressed / purified LC/A-nociceptin-H_N/A fusion protein family with variable spacer length product(s)

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Using the methodology outlined in Example 26, variants of the LC/A-CPN-H_N/A fusion consisting of GS10, GS30 and HX27 are purified from *E. coli* cell paste.

Samples from the purification of LC/A-CPN(GS10)-H_N/A, LC/A-CPN(GS15)-H_N/A, LC/A-CPN(GS25)-H_N/A, LC/A-CPN(GS30)-H_N/A and LC/A-CPN(HX27)-H_N/A were assessed by SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPBE-A. Top panel: M = benchmark molecular mass markers; S = total *E. coli* protein soluble fraction; FT = proteins that did not bind to the Ni²⁺-charged Sepharose column; FUSION = fusion protein eluted by the addition of imidazole. Bottom panel: Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 5 = purified final material post activation with Factor Xa (5 µl); Lane 6 = purified final material post activation with Factor Xa (10 µl); Lane 7 = purified final material post activation with Factor Xa (20 µl); Lane 8 = purified final material post activation with Factor Xa + DTT (5 µl); Lane 9 = purified final material post activation with Factor Xa + DTT (10 µl); Lane 10 = purified final material post activation with Factor Xa + DTT (20 µl).

Figure 13 - Inhibition of SP release and cleavage of SNAP-25 by CPN-A

Briefly, primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis and plotted against fusion concentration (dashed line). Material was also recovered for an analysis of substance P content using a specific EIA kit. Inhibition of substance P release is illustrated by the solid line. The fusion concentration required to achieve 50% maximal SNAP-25 cleavage is estimated to be 6.30±2.48 nM.

Figure 14 - Inhibition of SP release and cleavage of SNAP-25 over extended time periods after exposure of DRG to CPN-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A for 24 hours. Botulinum neurotoxin (BoNT/A) was used as a control. After this initial exposure, extracellular material was removed by washing, and the cells incubated at 37°C for varying periods of time. At specific time points, cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis and illustrated by the dotted lines. Material was also recovered for an analysis of substance P content using a specific EIA kit. Inhibition of substance P release is illustrated by the solid lines.

Figure 15 - Cleavage of SNAP-25 by CPNv-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. The fusion concentration required to achieve 50% maximal SNAP-25 cleavage is estimated to be 1.38 ± 0.36 nM.

Figure 16 - Cleavage of SNAP-25 over extended time periods after exposure of DRG to CPNv-A

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-A for 24 hours. CPN-A was used as a control. After this initial exposure, extracellular material was removed by washing, and the cells incubated at 37°C for varying periods of time. At specific time points, cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of

cleaved SNAP-25 was calculated by densitometric analysis.

Figure 17 - CPNv-A fusion-mediated displacement of [3H]-nociceptin binding

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The ability of nociceptin fusions to bind to the ORL₁ receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). It is clear that the LC/A-nociceptin variant-H_N/A fusion (labelled as CPNv-LHnA) is superior to the LC/A-nociceptin-H_N/A fusion (labelled as CPN-LHnA) at interacting with the ORL₁ receptor.

Figure 18 - Expressed / purified CPNv(Ek)-A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv(Ek)-A. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 4 = purified final material post activation with enterokinase (5 µl); Lane 5 = purified final material post activation with enterokinase (10 µl); Lane 6 = purified final material post activation with enterokinase (20 µl); Lane 7 = purified final material post activation with enterokinase + DTT (5 µl); Lane 8 = purified final material post activation with enterokinase + DTT (10 µl); Lane 9 = purified final material post activation with enterokinase + DTT (20 µl).

Figure 19 - Cleavage of SNAP-25 by CPNv(Ek)-A

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Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv(Ek)-A for 24 hours. Cellular proteins were separated by

SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. CPNv-A as prepared in Example 26 was used for comparison purposes. The percentage cleavage of SNAP-25 by CPNv(Ek)-A (labelled as En activated) and CPNv-A (labelled as Xa activated) are illustrated.

Figure 20 - Expressed / purified CPNv-C product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv-C. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 5 = purified material following second capture on Ni²⁺-charged Sepharose; Lane 6 = final purified material; Lane 7 = final purified material + DTT; Lane 8 = benchmark molecular mass markers.

Figure 21 - Cleavage of syntaxin by CPNv-C

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPNv-C for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-syntaxin to facilitate an assessment of syntaxin cleavage. The percentage of cleaved syntaxin was calculated by densitometric analysis. The fusion concentration required to achieve 50% maximal syntaxin cleavage is estimated to be 3.13±1.96 nM.

Figure 22 - CPN-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

The ability of an LC/A-nociceptin-H_N/A fusion (CPN/A) to inhibit capsaicin-induced mechanical allodynia was evaluated following subcutaneous intraplantar injection in

the rat hind paw. Test animals were evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat); after subcutaneous intraplantar treatment with CPN/A but before capsaicin (Pre-CAP); and following capsaicin challenge post-injection of CPN/A (average of responses at 15' and 30'; CAP). Capsaicin challenge was achieved by injection of 10 μ L of a 0.3% solution. Sample dilutions were prepared in 0.5% BSA/saline.

Figure 23 - CPN-A efficacy in the Streptozotocin (STZ)-Induced Peripheral Diabetic Neuropathy (Neuropathic Pain) model

Male Sprague-Dawley rats (250-300 g) are treated with 65 mg/kg STZ in citrate buffer (I.V.) and blood glucose and lipid are measured weekly to define the readiness of the model. Paw Withdrawal Threshold (PWT) is measured in response to a Von Frey filament stimulus series over a period of time. Allodynia is said to be established when the PWT on two consecutive test dates (separated by 1 week) measures below 6 g on the scale. At this point, rats are randomized to either a saline group (negative efficacy control), gabapentin group (positive efficacy control) or a test group (CPN/A). Test materials (20-25 μ l) are injected subcutaneously as a single injection (except gabapentin) and the PWT is measured at 1 day post-treatment and periodically thereafter over a 2 week period. Gabapentin (30 mg/kg i.p. @ 3 ml/kg injection volume) is injected daily, 2 hours prior to the start of PWT testing.

Figure 24 - CPNv-A efficacy in the Acute Capsaicin-Induced Mechanical Allodynia model

The ability of an LC/A-nociceptin variant-H_N/A fusion (CPNv/A) to inhibit capsaicin-induced mechanical allodynia was evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals were evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat), after subcutaneous

intraplantar treatment with CPNv/A but before capsaicin (Pre-CAP), and following capsaicin challenge post-injection of CPNv/A (average of responses at 15' and 30'; CAP). Capsaicin challenge was achieved by injection of 10 μ L of a 0.3% solution. Sample dilutions were prepared in 0.5% BSA/saline. These data are expressed as a normalized paw withdrawal frequency differential, in which the difference between the peak response (post-capsaicin) and the baseline response (pre-capsaicin) is expressed as a percentage. With this analysis, it can be seen that CPNv/A is more potent than CPN/A since a lower dose of CPNv/A is required to achieve similar analgesic effect to that seen with CPN/A.

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Figure 25 - Expressed / purified LC/A-CPLE-H_N/A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPLE-A. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 5 = purified material following second capture on Ni²⁺-charged Sepharose; Lane 6 = final purified material; Lane 7 = final purified material + DTT.

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Figure 26 - Expressed / purified LC/A-CPBE-H_N/A product

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPBE-A. Lane 1 = total *E. coli* protein soluble fraction; Lane 2 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 3 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 4 = purified final material post activation with Factor Xa (5 μ l); Lane 5 = purified final material post activation with Factor Xa (10 μ l); Lane 6 = purified final material post activation with Factor Xa (20 μ l); Lane 7 = purified final material post activation with Factor Xa + DTT (5 μ l); Lane 8 = purified final material

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post activation with Factor Xa + DTT (10 μ l); Lane 9 = purified final material post activation with Factor Xa + DTT (20 μ l); Lane 10 = benchmark molecular mass markers.

5 **Figure 27 - Expressed / purified CPOP-A product**

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPOP-A. Lane 1 = benchmark molecular mass
10 markers; Lane 2 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 3 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 4 = purified material following second capture on Ni²⁺-charged Sepharose; Lane 5 = purified final material post activation with Factor Xa (5 μ l); Lane 6 = purified final material post activation with Factor Xa (10 μ l); Lane 7 =
15 purified final material post-activation with Factor Xa (20 μ l); Lane 8 = purified final material post activation with Factor Xa + DTT (5 μ l); Lane 9 = purified final material post activation with Factor Xa + DTT (10 μ l); Lane 10 = purified final material post activation with Factor Xa + DTT (20 μ l).

20 **Figure 28 - Expressed / purified CPOPv-A product**

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPOPv-A. Lane 1 = benchmark molecular mass
25 markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = purified material following initial capture on Ni²⁺-charged Sepharose; Lane 4 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 5 = purified final material post activation with Factor Xa (5 μ l); Lane 6 = purified final material post activation with Factor Xa (10 μ l); Lane 7 = purified final material post activation with
30 Factor Xa (20 μ l); Lane 8 = purified final material post activation with Factor Xa + DTT (5 μ l); Lane 9 = purified final material post activation with Factor Xa + DTT (10 μ l); Lane 10 = purified final material post activation with Factor Xa + DTT (20 μ l).

Figure 29 - *In vitro* SNAP-25 cleavage in a DRG cell model

5 Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPOPv-A for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis.

10

Figure 30 - Expressed / purified CPNv-A-FXa-HT (removable his-tag)

Proteins were subjected to SDS-PAGE prior to staining with Coomassie Blue. The electrophoresis profile indicates purification of a disulphide-bonded di-chain species of the expected molecular mass of CPNv-A-FXa-HT. Lane 1 = benchmark molecular mass markers; Lane 2 = total *E. coli* protein soluble fraction; Lane 3 = Factor Xa treated material prior to final capture on Ni²⁺-charged Sepharose; Lane 4 = purified final material post activation with Factor Xa; Lane 5 = purified final material post activation with Factor Xa + DTT.

20

Figure 31 - *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by ligand competition assay

The ability of LC/A-nociceptin-H_N/A fusions of variable spacer length to bind to the ORL₁ receptor was assessed using a simple competition-based assay. Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of test material in the presence of 1 nM [3H]-nociceptin. The reduction in specific binding of the radiolabelled ligand was assessed by scintillation counting, and plotted in comparison to the efficacy of unlabelled ligand (Tocris nociceptin). The upper panel illustrates the displacement characteristics of the GS0, GS20, GS30 and Hx27 spacers, whilst the lower panel illustrates the displacement achieved by the GS10, GS15 and GS25 spaced fusion proteins. It is concluded that the GS0 and GS30

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spacers are ineffective, and the GS10 is poorly effective, at displacing nociceptin from the ORL1 receptor.

Figure 32 - *In vitro* efficacy of LC/A-nociceptin-H_N/A fusion proteins with variable spacer length, as assessed by *in vitro* SNAP-25 cleavage

Primary cultures of dorsal root ganglia (DRG) were exposed to varying concentrations of CPN-A (of variable spacer length) for 24 hours. Cellular proteins were separated by SDS-PAGE, Western blotted, and probed with anti-SNAP-25 to facilitate an assessment of SNAP-25 cleavage. The percentage of cleaved SNAP-25 was calculated by densitometric analysis. The poorly effective binding characteristics of the GS10 spaced fusion protein (see Figure 28) are reflected in the higher concentrations of fusion required to achieve cleavage of intracellular SNAP-25. GS0 and GS30 spaced fusion proteins were completely ineffective (date not shown). GS15, 20 and 25-spaced fusion proteins were similarly effective.

SEQ ID Nos

	SEQ ID1	DNA sequence of N[1-17]
20	SEQ ID2	Protein Sequence of N[1-17]
	SEQ ID3	DNA sequence of N[1-11]
	SEQ ID4	Protein sequence of N[1-11]
	SEQ ID5	DNA sequence of N[[Y10]1-11]
	SEQ ID6	Protein sequence of N[[Y10]1-11]
25	SEQ ID7	DNA sequence of N[[Y11]1-11]
	SEQ ID8	Protein sequence of N[[Y11]1-11]
	SEQ ID9	DNA sequence of N[[Y14]1-17]
	SEQ ID10	Protein sequence of N[[Y14]1-17]
	SEQ ID11	DNA sequence of N[1-13]
30	SEQ ID12	Protein sequence of N[1-13]
	SEQ ID13	DNA sequence of Nv (also known as N[[R14K15]1-17])
	SEQ ID14	Protein sequence of Nv (also known as N[[R14K15]1-17])

	SEQ ID15	DNA sequence of N[1-17]-LH _N /A fusion protein
	SEQ ID16	Protein sequence of N[1-17]-LH _N /A fusion protein
	SEQ ID17	DNA sequence of N[[Y11]1-11]-LHN/A fusion protein
	SEQ ID18	Protein sequence of N[[Y11]1-11]-LHN/A fusion protein
5	SEQ ID19	DNA sequence of N[1-13]-LHN/A fusion protein
	SEQ ID20	Protein sequence of N[1-13]-LHN/A fusion protein
	SEQ ID21	DNA sequence of LHN/A-N[1-17] fusion protein
	SEQ ID22	Protein sequence of LHN/A-N[1-17] fusion protein
	SEQ ID23	DNA sequence of LHN/C-N[1-11] fusion protein
10	SEQ ID24	Protein sequence of LHN/C-N[1-11] fusion protein
	SEQ ID25	DNA sequence of N[[Y14]1-17]-LHN/C fusion protein
	SEQ ID26	Protein sequence of N[[Y14]1-17]-LHN/C fusion protein
	SEQ ID27	DNA sequence of the LC/A
	SEQ ID28	DNA sequence of the H _N /A
15	SEQ ID29	DNA sequence of the LC/B
	SEQ ID30	DNA sequence of the H _N /B
	SEQ ID31	DNA sequence of the LC/C
	SEQ ID32	DNA sequence of the H _N /C
	SEQ ID33	DNA sequence of the CPN-A linker
20	SEQ ID34	DNA sequence of the A linker
	SEQ ID35	DNA sequence of the N-terminal presentation nociceptin insert
	SEQ ID36	DNA sequence of the CPN-C linker
	SEQ ID37	DNA sequence of the CPBE-A linker
	SEQ ID38	DNA sequence of the CPNvar-A linker
25	SEQ ID39	DNA sequence of the LC/A-CPN-H _N /A fusion
	SEQ ID40	Protein sequence of the LC/A-CPN-H _N /A fusion
	SEQ ID41	DNA sequence of the N-LC/A-H _N /A fusion
	SEQ ID42	Protein sequence of the N-LC/A-H _N /A fusion
	SEQ ID43	DNA sequence of the LC/C-CPN-H _N /C fusion
30	SEQ ID44	Protein sequence of the LC/C-CPN-H _N /C fusion
	SEQ ID45	DNA sequence of the LC/C-CPN-H _N /C (A-linker) fusion
	SEQ ID46	Protein sequence of the LC/C-CPN-H _N /C (A-linker) fusion

- SEQ ID47 DNA sequence of the LC/A-CPME-H_N/A fusion
- SEQ ID48 Protein sequence of the LC/A-CPME-H_N/A fusion
- SEQ ID49 DNA sequence of the LC/A-CPBE-H_N/A fusion
- ~~SEQ ID50 Protein sequence of the LC/A-CPBE-H_N/A fusion~~
- 5 SEQ ID51 DNA sequence of the LC/A-CPNv-H_N/A fusion
- SEQ ID52 Protein sequence of the LC/A-CPNv-H_N/A fusion
- SEQ ID53 DNA sequence of the LC/A-CPN[1-11]-HN/A fusion
- SEQ ID54 Protein sequence of the LC/A-CPN[1-11]-HN/A fusion
- SEQ ID55 DNA sequence of the LC/A-CPN[[Y10]1-11]-HN/A fusion
- 10 SEQ ID56 Protein sequence of the LC/A-CPN[[Y10]1-11]-HN/A fusion
- SEQ ID57 DNA sequence of the LC/A-CPN[[Y11]1-11]-HN/A fusion
- SEQ ID58 Protein sequence of the LC/A-CPN[[Y11]1-11]-HN/A fusion
- SEQ ID59 DNA sequence of the LC/A-CPN[[Y14]1-17]-HN/A fusion
- SEQ ID60 Protein sequence of the LC/A-CPN[[Y14]1-17]-HN/A fusion
- 15 SEQ ID61 DNA sequence of the LC/A-CPN[1-13]-HN/A fusion
- SEQ ID62 Protein sequence of the LC/A-CPN[1-13]-HN/A fusion
- SEQ ID63 DNA sequence of the nociceptin-spacer-LC/A-H_N/A fusion
- SEQ ID64 Protein sequence of the nociceptin-spacer-LC/A-H_N/A fusion
- SEQ ID65 DNA sequence of the CPN-A GS10 linker
- 20 SEQ ID66 DNA sequence of the CPN-A GS15 linker
- SEQ ID67 DNA sequence of the CPN-A GS25 linker
- SEQ ID68 DNA sequence of the CPN-A GS30 linker
- SEQ ID69 DNA sequence of the CPN-A HX27 linker
- SEQ ID70 DNA sequence of the LC/A-CPN(GS15)-H_N/A fusion
- 25 SEQ ID71 Protein sequence of the LC/A-CPN(GS15)-H_N/A fusion
- SEQ ID72 DNA sequence of the LC/A-CPN(GS25)-H_N/A fusion
- SEQ ID73 Protein sequence of the LC/A-CPN(GS25)-H_N/A fusion
- SEQ ID74 DNA sequence of the CPNvar-A Enterokinase activatable linker
- SEQ ID75 DNA sequence of the LC/A-CPNv(Ek)-H_N/A fusion
- 30 SEQ ID76 Protein sequence of the LC/A-CPNv(Ek)-H_N/A fusion
- SEQ ID77 DNA sequence of the CPNvar-A linker
- SEQ ID78 DNA sequence of the LC/C-CPNv-H_N/C fusion (act. A)

- SEQ ID79 Protein sequence of the LC/C-CPNv-H_N/C fusion (act. A)
SEQ ID80 DNA sequence of the LC/A-CPLE-H_N/A fusion
SEQ ID81 Protein sequence of the LC/A-CPLE-H_N/A fusion
SEQ ID82 DNA sequence of the LC/A-CPOP-H_N/A fusion
5 SEQ ID83 Protein sequence of the LC/A-CPOP-H_N/A fusion
SEQ ID84 DNA sequence of the LC/A-CPOPv-H_N/A fusion
SEQ ID85 Protein sequence of the LC/A-CPOPv-H_N/A fusion
SEQ ID86 DNA sequence of the IgA protease
SEQ ID87 DNA sequence of the IgA-CPNv-H_N/A fusion
10 SEQ ID88 Protein sequence of the IgA-CPNv-H_N/A fusion
SEQ ID89 DNA sequence of the FXa-HT
SEQ ID90 DNA sequence of the CPNv-A-FXa-HT
SEQ ID91 Protein sequence of the CPNv-A-FXa-HT fusion
SEQ ID92 DNA sequence of the DT translocation domain
15 SEQ ID93 DNA sequence of the CPLE-DT-A
SEQ ID94 Protein sequence of the CPLE-DT-A fusion
SEQ ID95 DNA sequence of the TeNT LC
SEQ ID96 DNA sequence of the CPNv-TeNT LC
SEQ ID97 Protein sequence of the CPNV-TeNT LC fusion
20 SEQ ID98 DNA sequence of the CPNvar-C linker
SEQ ID99 DNA sequence of the LC/C-CPNv-H_N/C fusion (act. C)
SEQ ID100 Protein sequence of the LC/C-CPNv-H_N/C fusion (act. C)

25 **Examples**

Example 1 – Confirmation of TM Agonist Activity by measuring release of substance P from neuronal cell cultures

30 *Materials*

Substance P EIA is obtained from R&D Systems, UK.

Methods

Primary neuronal cultures of eDRG are established as described previously (Duggan *et al.*, 2002). Substance P release from the cultures is assessed by EIA, essentially as described previously (Duggan *et al.*, 2002). The TM of interest is added to the neuronal cultures (established for at least 2 weeks prior to treatment); control cultures are performed in parallel by addition of vehicle in place of TM. Stimulated (100 mM KCl) and basal release, together with total cell lysate content, of substance P are obtained for both control and TM treated cultures. Substance P immunoreactivity is measured using Substance P Enzyme Immunoassay Kits (Cayman Chemical Company, USA or R&D Systems, UK) according to manufacturers' instructions.

The amount of Substance P released by the neuronal cells in the presence of the TM of interest is compared to the release obtained in the presence and absence of 100 mM KCl. Stimulation of Substance P release by the TM of interest above the basal release, establishes that the TM of interest is an "agonist ligand" as defined in this specification. If desired the stimulation of Substance P release by the TM of interest can be compared to a standard Substance P release-curve produced using the natural ORL-1 receptor ligand, nociceptin (Tocris).

Example 2 - Expression and purification of catalytically active LH_N/A

Materials

Synthetic DNA obtained from Sigma Genosys.

Restriction enzymes obtained from New England Biolabs.

Methods

The expression and purification of catalytically active LH_N/A was carried out essentially as described in Sutton *et al.*, (2005), *Prot. Express. Purif.*, 40, pp 31-41.

Briefly, DNA encoding the light chain plus 423 amino acids from the N-terminal of the heavy chain of BoNT/A was synthesised by Sigma-Genosys to produce a synthetic

LH_N/A gene with an *E. coli* codon bias. The linker region between the light chain and H_N domain was engineered to contain a Factor Xa cleavage site by splice-overlap extension PCR. Two PCR products were generated using primer pairs consisting of a long, mutagenic primer and a shorter, non-mutagenic primer:

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(5'-tccaaaactaaatctctgATAGAAGGTAGAAacaaagcgctgaacgac) with
(5'-CTTGATGTA CTCTGTGAACGTGCTC); and

10

(5'-gtcggtcagcgctttggtTCTACCTTCTATcagagatttagtttgga) with
(5'-ATGGAGTTCGTTAACAAACAGTTC).

The products from these two reactions were used as templates for the splice-overlap extension PCR. A further PCR reaction was set up to add *Bam*HI and *Sal*I sites at either end of the activatable recLH_N/A gene and these sites were used for insertion
15 into an Invitrogen gateway entry vector. The entry vector was then used, along with a gateway recombination site adapted pMAL c2x, in a LR clonase reaction to form pMAL c2x recLH_N/A. The pMAL c2x recLH_N/A was modified to incorporate a 6' HIS tag at the N-terminus of the MBP. This was achieved by the insertion of annealed oligonucleotides encoding the HIS tag into the *Nde*I site of pMAL.

20

The expression vector expressing LH_N/A was transformed into *E. coli* HMS174 or AD494(DE3) (Novagen). Cultures were grown in Terrific broth complex medium supplemented with ZnCl₂ (1 μM), ampicillin (100 μg/ml), 0.2% (w/v) glucose. Parameters for expression of all the constructs were initially determined in shake
25 flask cultures before transferring into 8 L fermentor systems. Starter cultures were grown for 16 hours at 37°C, 220 rpm and used to inoculate 1 L in which growth was continued at 37°C, 250 rpm. At an OD₆₀₀ nm of 0.6 the temperature was reduced to 25°C for 30 minutes before induction with 1 mM IPTG. Induction was continued for 4 hours before the cells were harvested and stored at -70°C.

30

Typically 16 g of cell paste was suspended in 160 ml PBS and lysed by sonication (MSE Soniprep 150). The resulting lysate was clarified by centrifugation prior

loading onto a 25 ml amylose column and eluted with 10 mM maltose in PBS. The eluant contained approx. 50% pure fusion protein and was treated with Factor Xa (1 unit Factor Xa /100 µg fusion protein; 20 hours; 26°C) to remove the HISMBP and cleave the LC-H_N junction to activate the protein. After incubation the sample was filtered (0.45 µm) and diluted two fold with water to give a 0.5 x PBS buffer composition. The cleaved, filtered and diluted recLH_N/A was processed through a Q Sepharose FF column (10 ml) and eluted with a step gradient of 80 mM NaCl containing HISMBP and 120 mM NaCl containing approx. 75% pure recLH_N/A. The addition of His tag to MBP overcame previous co-elution problems with LH_N/A and MBP. As a final polishing step to ensure complete removal of the HISMBP, the 120 mM NaCl elution from the Q Sepharose column was passed through a Nickel charged 5 ml HisTrap column (Amersham). The flow through from the HisTrap column contained approx. 95% pure recLH_N/A (see the Figures in Sutton *et al.*, (2005), Prot. Express. Purif., 40, pp 31-41 for an illustration of the purification scheme for LHN/A).

Example 3 - Expression and purification of catalytically active recombinant LH_N/B

The methodology described below will purify catalytically active LH_N/B protease from *E. coli* transformed with the appropriate plasmid encoding the LH_N/B polypeptide. It should be noted that various sequences of suitable LH_N/B polypeptides have been described in PCT/GB97/02273, granted US 6 461617 and US patent application 10/241596, incorporated herein by reference.

Methods

The coding region for LH_N/B is inserted in-frame to the 3' of the gene encoding maltose binding protein (MBP) in the expression vector pMAL (New England Biolabs) to create pMAL- c2x-LH_N/B. In this construct, the expressed MBP and LH_N/B polypeptides are separated by a Factor Xa cleavage site, and the LC and H_N domains are separated by a peptide that is susceptible to cleavage with enterokinase. The expression clone is termed pMAL-c2X-synLH_N/B.

pMAL-c2X-synLH_N/B is transformed into *E. coli* HMS174 and cultured in Terrific broth complex medium in 8 L fermentor systems. Pre-induction bacterial growth is maintained at 37°C to an OD_{600 nm} of 5.0, at which stage expression of recMBP-LH_N/B is induced by addition of IPTG to 0.5 mM and a reduction in temperature to 30°C. After four hours at 30°C the bacteria are harvested by centrifugation and the resulting paste stored at -70°C.

The cell paste is resuspended in 20 mM Hepes pH 7.2, 125 mM NaCl, 1 μM ZnCl₂ and cell disruption achieved using an APV-Gaulin lab model 1000 homogeniser or a MSE Soniprep 150 sonicator. The resulting suspension is clarified by centrifugation prior to purification.

Following cell disruption, the MBP-fusion protein is captured either on an amylose affinity resin in 20 mM Hepes pH 7.2, 125 mM NaCl, 1 μM ZnCl₂, or on a Q-Sepharose FF anion-exchange resin in 50 mM Hepes pH 7.2, 1 μM ZnCl₂ with no salt. A single peak is eluted from the amylose resin in the same buffer plus 10 mM maltose and from the Q-Sepharose in 150-200 mM salt. Cleavage of the MBP-LH_N/B junction is completed in an 18 hours incubation step at 22°C with Factor Xa (NEB) at 1 U/50 μg fusion protein. A substrate (MBP-LH_N/B) concentration of at least 4 mg/ml is desirable for efficient cleavage to take place.

The cleaved protein is diluted with 20 mM Hepes to a buffer composition of 20 mM Hepes, 25 mM NaCl, 1 μM ZnCl₂, pH 7.2 and processed through a Q Sepharose column to separate the MBP from LH_N/B. The LH_N/B is eluted from the Q-Sepharose column with 120-170 mM salt. The linker between the light chain and H_N domain is then nicked by incubation with enterokinase at 1 U/100 μg of LH_N/B at 22°C for 16 hours. Finally, the enterokinase is separated from the nicked LH_N/B and other contaminating proteins on a Benzamidine Sepharose column, the enzyme preferentially binding to the resin over an incubation of 30 minutes at 4°C. Purified LH_N/B is stored at -20°C until required. See Figure 1 for an illustration of the purification scheme for recLH_N/B.

Example 4 - Expression and purification of catalytically active recombinant LH_N/C

The coding region for LH_N/C is inserted in-frame to the 3' of the gene encoding maltose binding protein (MBP) in the expression vector pMAL (New England Biolabs) to create pMAL-c2x-LH_N/C. In this construct the expressed MBP and LH_N/C polypeptides are separated by a Factor Xa cleavage site.

pMAL-c2x-LH_N/C is transformed into *E. coli* AD494 (DE3, IRL) and cultured in Terrific broth complex medium in 8 L fermentor systems. Pre-induction bacterial growth are maintained at 30°C to an OD600 nm of 8.0, at which stage expression of recMBP-c2x-LH_N/C is induced by addition of IPTG to 0.5 mM and a reduction in temperature of culture to 25°C. After 4 hours at 25°C the bacteria are harvested by centrifugation and the resulting paste stored at -70°C.

The cell paste is resuspended in 50 mM Hepes pH 7.2, 1 μM ZnCl₂ at 1:6 (w/v) and cell disruption is achieved using an APV-Gaulin lab model 1000 homogeniser or a MSE Soniprep 150 sonicator. The resulting suspension is clarified by centrifugation prior to purification.

5

Following cell disruption and clarification, the MBP-fusion protein is separated on a Q-Sepharose Fast Flow anion-exchange resin in 50 mM Hepes pH 7.2, 1 μM ZnCl₂ and eluted with the same buffer plus 100 mM NaCl. A double point cleavage is performed at the MBP-LH_N/C junction and the H_N-LC linker in a single incubation step with Factor Xa. The reaction is completed in a 16-hour incubation step at 22°C with Factor Xa (NEB) at 1 U/100 μg fusion protein. The cleaved protein is diluted with 20 mM Hepes to a buffer composition of 20 mM Hepes, 25 mM NaCl, pH 7.2 and processed through a second Q-Sepharose column to separate the MBP from LH_N/C. Activated (disulphide-bonded cleaved linker) LH_N/C is eluted from the Q-Sepharose column by a salt gradient (20 mM Hepes, 500 mM NaCl, 1 μM ZnCl₂, pH 7.2) in 120-170 mM salt. See Figure 2 for an illustration of the purification of

10
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LH_N/C.

Example 5 - Production of a chemical conjugate of nociceptin and LH_N/A

5 *Materials*

C-terminally extended nociceptin peptide obtained from Sigma Genosys.

Conjugation chemicals obtained from Pierce.

Methods

10 In order to couple the nociceptin peptide via a C-terminal Cys, the peptide was first synthesised (by standard procedures, commercially obtainable) to include a Cys as the final C-terminal amino acid.

This peptide was then used as the second component in a sulphhydryl based
15 coupling reaction as described below (see also previous publications WO 99/17806 and WO 96/33273 and Duggan *et al.*, (2002), *J. Biol. Chem.* 277, 24846-34852 and Chaddock *et al.*, (2000), *Infect Immun.*, 68, 2587-2593).

Sulphydryl based coupling reaction

20 Briefly, approximately two reactive leaving groups were introduced into LH_N/A (5 mg/ml in phosphate-buffered saline) by reaction with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP).

Derivatised material was isolated from excess SPDP by size exclusion
25 chromatography. Reconstituted cysteine-tagged nociceptin ligand was mixed with the derivatised LH_N/A in a 4:1 molar ratio, and incubated at room temperature for 1 hour with gentle agitation in order to create a chemical conjugate through a reducible covalent disulphide bond. Initial fractionation of the conjugate mixture to remove unconjugated peptide was performed by size exclusion chromatography
30 (Superose-12, or Superdex G-200 depending on scale of conjugation).

Example 6 - Production of a chemical conjugate of nociceptin and LH_N/B*Materials*

5 C-terminally extended nociceptin peptide obtained from Sigma Genosys.
Conjugation chemicals obtained from Pierce.

Methods

10 Lyophilised nociceptin was dissolved by the addition of water and dialysed into MES
buffer (0.1 M MES, 0.1 M NaCl, pH 5.0). To this solution (at a concentration of about
0.3 mg/ml) was added PDPH (100 mg/ml in DMF) to a final concentration of
1 mg/ml. After mixing, solid EDAC was added to produce a final concentration of
about 0.2 mg/ml. The reaction was allowed to proceed for at least 30 minutes at
15 room temperature. Excess PDPH was then removed by desalting over a PD-10
column (Pharmacia) previously equilibrated with MES buffer.

An amount of LH_N/B equivalent to half the weight of nociceptin used dissolved in
triethanolamine buffer (0.02 M triethanolamine/HCl, 0.1 M sodium chloride, pH 7.8)
at a concentration of about 1 mg/ml, was reacted with Traut's reagent (100 mM stock
20 solution in 1 M triethanolamine/HCl, pH 8.0) at a final concentration of 2 mM. After
1 hour, the LH_N/B was desalted into PBSE (phosphate buffered saline with 1 mM
EDTA) using a PD-10 column (Pharmacia). The protein peak from the column
eluate was concentrated using a Microcon 50 (Amicon) to a concentration of about
2 mg/ml.

25

The derivatised nociceptin was subjected to a final concentration step resulting in a
reduction in volume to less than 10% of the starting volume and then mixed with the
derivatised LH_N/B overnight at room temperature. The products of the reaction were
analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-
30 sulphate (SDS-PAGE).

The conjugate resulting from the above reaction was partially purified by size

exclusion chromatography over Bio-Gel P-100 (BioRad). The elution profile was followed by measuring the optical density at 280 nm and SDS-PAGE analysis of the fractions. This allowed the separation of conjugate from free nociceptin and by-products of the reaction.

5

Example 7 - Production of a chemical conjugate of nociceptin 1-11 and LH_N/B

Materials

C-terminally extended nociceptin 1-11 peptide obtained from Sigma Genosys.

10 Conjugation chemicals obtained from Pierce.

Methods

In order to couple the nociceptin 1-11 peptide via a C-terminal Cys, the peptide was first synthesised (by standard procedures, commercially obtainable) to include a

15 Cys as the final C-terminal amino acid.

This peptide was then used as the second component in a sulphydryl based coupling reaction as described in Example 5.

20 Example 8 - Production of a chemical conjugate of nociceptin N[[Y14]1-17] and LH_N/C

Materials

C-terminally extended nociceptin N[[Y14]1-17] peptide obtained from Sigma

25 Genosys.

Conjugation chemicals obtained from Pierce.

Methods

In order to couple the peptide via a C-terminal Cys, the peptide was first synthesised
30 (by standard procedures, commercially obtainable) to include a Cys as the final C-terminal amino acid.

This peptide was then used as the second component in a sulphhydryl based coupling reaction as described in Example 5.

Example 9 - Recombinant production of a single polypeptide fusion of nociceptin-LH_N/A (SEQ ID15 and SEQ ID16)

The DNA sequence for the nociceptin-LH_N/A was designed by back translation of the LC/A, H_N/A, and nociceptin amino acid sequences. The complete ORF containing the nociceptin-LC/A-activation loop-H_N/A sequence was assembled within standard DNA sequence manipulation software (EditSeq). The activation loop between the LC/A cysteine and the H_N/A cysteine (CVRGIITSKTKSLDKGYNKALNDLC) was modified to incorporate a Factor Xa protease recognition site.

Restriction sites appropriate to facilitate cloning into the required expression vector (for example BamHI/Sall) were incorporated at the 5' and 3' ends respectively of the sequence maintaining the correct reading frame. The DNA sequence was screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that were found to be common to those required by the cloning system were removed manually from the proposed coding sequence ensuring common *E. coli* codon usage was maintained. *E. coli* codon usage was assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004).

This optimised DNA sequence containing the nociceptin-LC/A-activation loop-H_N/A open reading frame (ORF) was then commercially synthesized and provided in the pCR 4 vector.

The DNA encoding the nociceptin-LH_N/A fusion was isolated from pCR 4 and transferred into pMAL vector backbone to facilitate protein expression. The resultant pMAL NO-LHN/A vector was transformed into competent *E. coli* BL21 and correct

transformants selected. A single colony of pMAL NO-LH_N/A was grown in Terrific broth complex medium supplemented with ZnCl₂ (1 mM), ampicillin (100 µg/ml), 0.2% (w/v) glucose. Expression of the insert was induced by the addition of IPTG (0.1 mM) and the culture maintained at 16°C for 16 hours. After this period of expression the bacteria were isolated by centrifugation and the cell pellet stored at -20°C until use.

10 g of *E. coli* BL21 cell paste was defrosted in a falcon tube containing 25 ml 50 mM HEPES, pH 7.2, 200 mM NaCl. The thawed cell paste was made up to 80 ml with 50 mM HEPES, pH 7.2, 200 mM NaCl and sonicated on ice 30 seconds on, 30 seconds off for 10 cycles at a power of 22 microns ensuring the sample remained cool. The lysed cells were centrifuged at 18 000 rpm, 4°C for 30 minutes. The supernatant was loaded onto a 0.1 M NiSO₄ charged chelating column (20-30 ml column is sufficient) and equilibrated with 50 mM HEPES, pH 7.2, 200 mM NaCl.

Using a step gradient of 10 and 40 mM imidazol, the non-specific bound protein was washed away and the fusion protein eluted with 100 mM imidazol. The eluted fusion protein was dialysed against 5 L of 50 mM HEPES, pH 7.2, 200 mM NaCl at 4°C overnight and the OD of the dialysed fusion protein measured. 1 unit of Factor Xa was added per 100 µg fusion protein and incubated at 25°C static overnight. The cleavage mixture was loaded onto a 0.1 M NiSO₄ charged Chelating column (20-30 ml column is sufficient) and equilibrated with 50 mM HEPES, pH 7.2, 200 mM NaCl.

Using a step gradient of 10 and 40 mM imidazol, the non-specific bound protein was washed away and the fusion protein eluted with 100 mM imidazol. The eluted fusion protein was dialysed against 5 L of 50 mM HEPES, pH 7.2, 200 mM NaCl at 4°C overnight and the fusion concentrated to about 2 mg/ml, aliquoted and stored at -20°C.

30

Figure 3 shows the SDS-PAGE analysis of expression and purification of N[1-17]-LH_N/A .

Example 10 – Recombinant production of a single polypeptide fusion of (nociceptin 1-11)-LH_N/B

5 The DNA sequence for the (nociceptin 1-11)-LH_N/B was designed by back translation of the LC/B, H_N/B, and nociceptin 1-11 amino acid sequences. The complete ORF containing the (nociceptin1-11)-LC/B-activation loop-H_N/B sequence was assembled within standard DNA sequence manipulation software (EditSeq). The activation loop between the LC/B cysteine and the H_N/B cysteine was modified
10 to incorporate a Factor Xa protease recognition site.

The recombinant fusion protein was then produced essentially as described in Example 9.

15 **Example 11 – Recombinant production of a single polypeptide fusion of (nociceptin N[[Y14]1-17]) -LH_N/C (SEQ ID25 and SEQ ID26)**

The DNA sequence for the nociceptin N[[Y14]1-17] was designed by back translation of the LC/C, H_N/C, and nociceptin N[[Y14]1-17] amino acid sequences. The
20 complete ORF containing the (nociceptin N[[Y14]1-17])-LC/C-activation loop-H_N/C sequence was assembled within standard DNA sequence manipulation software (EditSeq). The activation loop between the LC/C cysteine and the H_N/C cysteine was modified to incorporate a Factor Xa protease recognition site.

25 The recombinant fusion protein was then produced essentially as described in Example 9.

30 **Example 12 – Recombinant production of a single polypeptide fusion of LH_N/C-(nociceptin 1-11) (SEQ ID23 and SEQ ID24)**

The DNA sequence for the LH_N/C-(nociceptin 1-11) was designed by back

translation of the LC/C, H_N/C and nociceptin 1-11 amino acid sequences. The complete ORF (SEQ ID23) containing the LC/C-activation loop-H_N/C-flexible spacer-(nociceptin 1-11) was assembled within standard DNA sequence manipulation software (EditSeq).

5

The recombinant fusion protein (SEQ ID24) was then produced essentially as described in Example 9.

Example 13 - Production of a conjugate for delivery of DNA encoding LC/C into a cell

10

The construction of a nociceptin-H_N-[LC/C] conjugate is described below, where [LC/C] represents the polylysine condensed DNA encoding the light chain of botulinum neurotoxin type C.

15

Materials

SPDP is from Pierce Chemical Co.

Additional reagents are obtained from Sigma Ltd.

Methods

20

Using a plasmid containing the gene encoding LC/C under the control of a CMV (immediate early) promoter, condensation of DNA was achieved using SPDP-derivatised polylysine to a ratio of 2 DNA to 1 polylysine. Conjugates were then prepared by mixing condensed DNA (0.4 mg/ml) with H_N-nociceptin (100 µg/ml) for 25 16 h at 25°C. The SPDP-derivatised polylysine and the free -SH group present on the H_N domain combine to facilitate covalent attachment of the DNA and protein.

Example 14 - Production of a conjugate for delivery of DNA encoding LC/B into a cell

30

The construction of a (nociceptin 1-11)-H_N-[LC/B] conjugate is described below, where [LC/B] represents the polylysine condensed DNA encoding the light chain of

botulinum neurotoxin type B.

Materials

SPDP is from Pierce Chemical Co.

5 Additional reagents are obtained from Sigma Ltd.

Methods

Using a plasmid containing the gene encoding LC/B under the control of a CMV (immediate early) promoter, condensation of DNA was achieved using SPDP-
10 derivatised polylysine to a ratio of 2 DNA to 1 polylysine. Conjugates were then prepared by mixing condensed DNA (0.4 mg/ml) with H_N-(nociceptin 1-11) (100 µg/ml) for 16 h at 25°C. The SPDP-derivatised polylysine and the free -SH group present on the H_N domain combine to facilitate covalent attachment of the DNA and protein.

15 —

Example 15 – Assessment of the activity of nociceptin-LH_N/A in substance P releasing neuronal cells

Using methodology described in Duggan *et al.*, (2002, J. Biol. Chem., 277, 34846-
20 34852), the activity of nociceptin-LH_N/A in substance P releasing neuronal cells was assessed.

Nociceptin-LH_N/A fusion protein was applied to 2-week old dorsal root ganglia neuronal cultures, and incubated at 37°C for 16 hours. Following the incubation, the
25 media was removed and the ability of the cells to undergo stimulated release of substance P (SP) was assessed.

The release of SP from the neuronal cells incubated with the nociceptin-LH_N/A fusion protein was assayed in comparison to (i) LH_N/A-only treated cells and (ii) cells
30 treated with media alone. This allowed the % inhibition of substance P from the eDRG to be calculated. The ability of the nociceptin-LH_N/A fusion protein to inhibit SP release (relative to cells treated with media alone) was reported in Table 1. The

data represent the mean of 3 determinations:

Table 1

Test Material (μM)	nociceptin-LH _N /A fusion protein	LH _N /A-only
	% Inhibition	% Inhibition
1.0	47.3	25.6
0.1	13.8	-11.5

5

Example 16 - Confirmation of ORL₁ receptor activation by measuring forskolin-stimulated cAMP production

Confirmation that a given TM is acting via the ORL₁ receptor is provided by the following test, in which the TMs ability to inhibit forskolin-stimulated cAMP production is assessed.

10

Materials

[³H]adenine and [¹⁴C]cAMP are obtained from GE Healthcare

15

Methods

The test is conducted essentially as described previously by Meunier *et al.* [Isolation and structure of the endogenous agonist of opioid receptor-like ORL₁ receptor. Nature 377: 532-535, 1995] in intact transfected-CHO cells plated on 24-well plastic plates.

20

To the cells is added [³H]adenine (1.0 μCi) in 0.4 ml of culture medium. The cells remain at 37°C for 2 h to allow the adenine to incorporate into the intracellular ATP pool. After 2 h, the cells are washed once with incubation buffer containing: 130 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 10 mM glucose, 1 mg/ml bovine serum albumin and 25 mM HEPES, pH 7.4, and replaced with buffer containing forskolin (10 μM) and isobutylmethylxanthine (50 μM) with or without the

25

TM of interest. After 10 min., the medium is aspirated and replaced with 0.5 ml, 0.2 M HCl. Approximately 1000 cpm of [¹⁴C]cAMP is added to each well and used as an internal standard. The contents of the wells are then transferred to columns of 0.65 g dry-alumina powder. The columns are eluted with 4 ml of 5 mM HCl, 0.5 ml of 0.1 M ammonium acetate, then two additional millilitres of ammonium acetate. The final eluate is collected into scintillation vials and counted for ¹⁴C and tritium. Amounts collected are corrected for recovery of [¹⁴C]cAMP. TMs that are agonists at the ORL₁ receptor cause a reduction in the level of cAMP produced in response to forskolin.

10

Example 17 - Confirmation of ORL₁ receptor activation using a GTPγS binding functional assay

Confirmation that a given TM is acting via the ORL₁ receptor is also provided by the following test, a GTPγS binding functional assay.

15

Materials

[³⁵S]GTPγS is obtained from GE Healthcare

Wheatgerm agglutinin-coated (SPA) beads are obtained from GE Healthcare

20

Methods

This assay is carried out essentially as described by Traynor and Nahorski [Modulation by μ-opioid agonists of guanosine-5'-O-(3-[³⁵S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 47: 848-854, 1995].

25

Cells are scraped from tissue culture dishes into 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid, then centrifuged at 500 × g for 10 min. Cells are resuspended in this buffer and homogenized with a Polytron Homogenizer.

30

The homogenate is centrifuged at 27,000 × g for 15 min., and the pellet resuspended in buffer A, containing: 20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl,

pH 7.4. The suspension is recentrifuged at 20,000 × *g* and suspended once more in buffer A. For the binding assay, membranes (8-15 µg protein) are incubated with [³⁵S]GTP S (50 pM), GDP (10 µM), with and without the TM of interest, in a total volume of 1.0 ml, for 60 min. at 25°C. Samples are filtered over glass fibre filters and counted as described for the binding assays.

Example 18 - Preparation of a LC/A and H_N/A backbone clones

The following procedure creates the LC and H_N fragments for use as the component backbone for multidomain fusion expression. This example is based on preparation of a serotype A based clone (SEQ ID27 and SEQ ID28), though the procedures and methods are equally applicable to the other serotypes [illustrated by the sequence listing for serotype B (SEQ ID29 and SEQ ID30) and serotype C (SEQ ID31 and SEQ ID32)].

15

Preparation of cloning and expression vectors

pCR 4 (Invitrogen) is the chosen standard cloning vector, selected due to the lack of restriction sequences within the vector and adjacent sequencing primer sites for easy construct confirmation. The expression vector is based on the pMAL (NEB) expression vector, which has the desired restriction sequences within the multiple cloning site in the correct orientation for construct insertion (*Bam*HI-*Sal*I-*Pst*I-*Hind*III).

20

A fragment of the expression vector has been removed to create a non-mobilisable plasmid and a variety of different fusion tags have been inserted to increase purification options.

25

Preparation of protease (e.g. LC/A) insert

The LC/A (SEQ ID27) is created by one of two ways:

The DNA sequence is designed by back translation of the LC/A amino acid sequence [obtained from freely available database sources such as GenBank (accession number P10845) or Swissprot (accession locus BXA1_CLOBO) using one of a variety of reverse translation software tools (for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)].

30

*Bam*HI/*Sal*I recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence, maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction-enzyme cleavage sequences incorporated during the back translation.

5 Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to

10 published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence containing the LC/A open reading frame (ORF) is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

15 The alternative method is to use PCR amplification from an existing DNA sequence with *Bam*HI and *Sal*I restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. Complementary oligonucleotide primers are chemically synthesised by a supplier (for example MWG or Sigma-Genosys), so that each pair has the ability to hybridize to the opposite strands (3' ends pointing "towards" each

20 other) flanking the stretch of *Clostridium* target DNA, one oligonucleotide for each of the two DNA strands. To generate a PCR product the pair of short oligonucleotide primers specific for the *Clostridium* DNA sequence are mixed with the *Clostridium* DNA template and other reaction components and placed in a machine (the 'PCR machine') that can change the incubation temperature of the reaction tube

25 automatically, cycling between approximately 94°C (for denaturation), 55°C (for oligonucleotide annealing), and 72°C (for synthesis). Other reagents required for amplification of a PCR product include a DNA polymerase (such as *Taq* or *Pfu* polymerase), each of the four nucleotide dNTP building blocks of DNA in equimolar amounts (50-200 µM) and a buffer appropriate for the enzyme optimised for Mg²⁺

30 concentration (0.5-5 mM).

The amplification product is cloned into pCR 4 using either, TOPO TA cloning for *Taq* PCR products or Zero Blunt TOPO cloning for *Pfu* PCR products (both kits commercially available from Invitrogen). The resultant clone is checked by sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis [for example, using Quickchange (Stratagene Inc.)].

Preparation of translocation (e.g. H_N) insert

The H_N/A (SEQ ID28) is created by one of two ways:

10 The DNA sequence is designed by back translation of the H_N/A amino acid sequence [obtained from freely available database sources such as GenBank (accession number P10845) or Swissprot (accession locus BXA1_CLOBO)] using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)].

15 A *Pst*I restriction sequence added to the N-terminus and *Xba*I-stop codon-*Hind*III to the C-terminus ensuring the correct reading frame is maintained. The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any sequences that are found to be common to those required by the cloning

20 system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September

25 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The alternative method is to use PCR amplification from an existing DNA sequence

30 with *Pst*I and *Xba*I-stop codon-*Hind*III restriction enzyme sequences incorporated into the 5' and 3' PCR primers respectively. The PCR amplification is performed as described above. The PCR product is inserted into pCR 4 vector and checked by

sequencing. Any additional restriction sequences which are not compatible with the cloning system are then removed using site directed mutagenesis [for example using Quickchange (Stratagene Inc.)].

5 **Example 19 – Preparation of a LC/A-nociceptin-H_N/A fusion protein (nociceptin is N-terminal of the H_N-chain)**

Preparation of linker-nociceptin-spacer insert

The LC-H_N linker can be designed from first principle, using the existing sequence
10 information for the linker as the template. For example, the serotype A linker (in this case defined as the inter-domain polypeptide region that exists between the cysteines of the disulphide bridge between LC and H_N) is 23 amino acids long and has the sequence VRGIITSKTKSLDKGYNKALNDL. Within this sequence, it is understood that proteolytic activation in nature leads to an H_N domain that has an N-
15 terminus of the sequence ALNDL. This sequence information is freely available from available database sources such as GenBank (accession number P10845) or Swissprot (accession locus BXA1_CLOBO). Into this linker a Factor Xa site, nociceptin and spacer are incorporated; and using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation
20 (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the linker-ligand-spacer region is determined. Restriction sites are then incorporated into the DNA sequence and can be arranged as *Bam*HI-*Sal*I-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID33).

It is important to ensure the correct reading frame is maintained for the spacer,
25 nociceptin and restriction sequences and that the *Xba*I sequence is not preceded by the bases, TC, which would result on DAM methylation. The DNA sequence is screened for restriction sequence incorporation, and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software
30 programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example, GenBank Release 143, 13 September 2004). This optimised

DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

~~Preparation of the LC/A-nociceptin-H_N/A fusion~~

5 In order to create the LC-linker-nociceptin-spacer-H_N construct (SEQ ID39), the pCR 4 vector encoding the linker (SEQ ID33) is cleaved with *Bam*HI + *Sall* restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID27) cleaved with *Bam*HI + *Sall*. The resulting plasmid DNA is then cleaved with *Pst*I + *Xba*I restriction enzymes and serves as the
10 recipient vector for the insertion and ligation of the H_N/A DNA (SEQ ID28) cleaved with *Pst*I + *Xba*I. The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID39) for transfer into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID40.

15 **Example 20 – Preparation of a nociceptin-LC/A-H_N/A fusion protein (nociceptin is N-terminal of the LC-chain)**

The LC/A-H_N/A backbone is constructed as described in Example 19 using the synthesised A serotype linker with the addition of a Factor Xa site for activation,
20 arranged as *Bam*HI-*Sall*-linker-protease site-linker-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID34). The LC/A-H_N/A backbone and the synthesised N-terminal presentation nociceptin insert (SEQ ID35) are cleaved with *Bam*HI + *Hind*III restriction enzymes, gel purified and ligated together to create a nociceptin-spacer-LC-linker-H_N. The ORF (SEQ ID41) is then cut out using restriction enzymes *Ava*I + *Xba*I for transfer
25 into expression vectors for expression to result in a fusion protein of the sequence illustrated in SEQ ID42.

Example 21 – Preparation of a LC/C-nociceptin-H_N/C fusion protein

30 Following the methods used in Examples 1 and 2, the LC/C (SEQ ID31) and H_N/C (SEQ ID32) are created and inserted into the C serotype linker arranged as *Bam*HI-*Sall*-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III

(SEQ ID36). The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID43) for expression as a protein of the sequence illustrated in SEQ ID44.

5 **Example 22 - Preparation of a LC/C-nociceptin-H_N/C fusion protein with a serotype A activation sequence**

Following the methods used in Examples 1 and 2, the LC/C (SEQ ID31) and H_N/C (SEQ ID32) are created and inserted into the A serotype linker arranged as *Bam*HI-*Sall*-linker-protease site-nociceptin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID33). The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID45) for expression as a protein of the sequence illustrated in SEQ ID46.

Example 23 - Preparation of a LC/A-met enkephalin-H_N/A fusion protein

15 Due to the small, five-amino acid, size of the met-enkephalin ligand the LC/A-met enkephalin-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID39) as a template. Oligonucleotides are designed encoding the YGGFM met-enkephalin peptide, ensuring standard *E.coli* codon usage is maintained and no additional
20 restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID39) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC-linker-met enkephalin-spacer-H_N ORF (SEQ ID47) for expression as a protein of the sequence illustrated in SEQ ID48.

25

Example 24 - Preparation of a LC/A-β endorphin-H_N/A fusion protein

Following the methods used in Examples 1 and 2, the LC/A (SEQ ID27) and H_N/A (SEQ ID28) are created and inserted into the A serotype β endorphin linker arranged
30 as *Bam*HI-*Sall*-linker-protease site-β endorphin-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID37). The final construct contains the LC-linker-β endorphin-

spacer-H_N ORF (SEQ ID49) for expression as a protein of the sequence illustrated in SEQ ID50.

Example 25 -- Preparation of a LC/A-nociceptin variant-H_N/A fusion protein

5

Following the methods used in Examples 1 and 2, the LC/A (SEQ ID27) and H_N/A (SEQ ID28) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sall*-linker-protease site-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID38). The final construct contains the LC-
10 linker-nociceptin variant-spacer-H_N ORF (SEQ ID51) for expression as a protein of the sequence illustrated in SEQ ID52.

Example 26 – Purification method for LC/A-nociceptin-H_N/A fusion protein

15 --Defrost falcon tube containing 25 ml 50 mM HEPES pH 7.2, 200 mM NaCl and approximately 10 g of *E. coli* BL21 cell paste. Make the thawed cell paste up to 80 ml with 50 mM HEPES pH 7.2, 200 mM NaCl and sonicate on ice 30 seconds on, 30 seconds off for 10 cycles at a power of 22 microns ensuring the sample remains cool. Spin the lysed cells at 18 000 rpm, 4°C for 30 minutes. Load the supernatant
20 onto a 0.1 M NiSO₄ charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2, 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazol, wash away the non-specific bound protein and elute the fusion protein with 100 mM imidazol. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2, 200 mM NaCl at 4°C overnight and measure the OD of the
25 dialysed fusion protein. Add 1 unit of factor Xa per 100 µg fusion protein and Incubate at 25°C static overnight. Load onto a 0.1 M NiSO₄ charged Chelating column (20-30 ml column is sufficient) equilibrated with 50 mM HEPES pH 7.2, 200 mM NaCl. Wash column to baseline with 50 mM HEPES pH 7.2, 200 mM NaCl. Using a step gradient of 10 and 40 mM imidazol, wash away the non-specific bound
30 protein and elute the fusion protein with 100 mM imidazol. Dialyse the eluted fusion protein against 5 L of 50 mM HEPES pH 7.2, 200 mM NaCl at 4°C overnight and

concentrate the fusion to about 2 mg/ml, aliquot sample and freeze at -20°C. Test purified protein using OD, BCA, purity analysis and SNAP-25 assessments.

Example 27 – Preparation of a LC/A-nociceptin-H_N/A fusion protein (nociceptin is N-terminal of the H_N-chain)

The linker-nociceptin-spacer insert is prepared as described in Example 19.

Preparation of the LC/A-nociceptin-H_N/A fusion

In order to create the LC-linker-nociceptin-spacer-H_N construct (SEQ ID39), the pCR 4 vector encoding the linker (SEQ ID33) is cleaved with *Bam*HI + *Sall* restriction enzymes. This cleaved vector then serves as the recipient for insertion and ligation of the LC/A DNA (SEQ ID27) also cleaved with *Bam*HI + *Sall*. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sall*, *Pst*I, and *Hind*III such as the pMAL vector (NEB). The H_N/A DNA (SEQ ID28) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The final construct contains the LC-linker-nociceptin-spacer-H_N ORF (SEQ ID39) for expression as a protein of the sequence illustrated in SEQ ID40.

Example 28 – Preparation of a nociceptin-LC/A-H_N/A fusion protein (nociceptin is N-terminal of the LC-chain)

In order to create the nociceptin-spacer-LC/A-H_N/A construct, an A serotype linker with the addition of a Factor Xa site for activation, arranged as *Bam*HI-*Sall*-linker-protease site-linker-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID34) is synthesised as described in Example 27. The pCR 4 vector encoding the linker is cleaved with *Bam*HI + *Sall* restriction enzymes. This cleaved vector then serves as the recipient for insertion and ligation of the LC/A DNA (SEQ ID27) also cleaved with *Bam*HI + *Sall*. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction

enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing the synthesised N-terminal presentation nociceptin insert (SEQ ID35).

This construct is then cleaved with *AvaI* + *HindIII* and inserted into an expression vector such as the pMAL-plasmid (NEB). The H_N/A DNA (SEQ ID28) is then cleaved

5 with *PstI* + *HindIII* restriction enzymes and inserted into the similarly cleaved pMAL-nociceptin-LC/A-linker construct. The final construct contains the nociceptin-spacer-LC/A-H_N/A ORF (SEQ ID63) for expression as a protein of the sequence illustrated in SEQ ID64.

10 **Example 29 - Preparation and purification of an LC/A-nociceptin-H_N/A fusion protein family with variable spacer length**

Using the same strategy as employed in Example 19, a range of DNA linkers were prepared that encoded nociceptin and variable spacer content. Using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the linker-ligand-spacer region is determined. Restriction sites are then incorporated into the DNA sequence and can be arranged as *BamHI-Sall-linker-protease site-nociceptin-NheI-spacer-SpeI-PstI-XbaI-stop codon-HindIII* (SEQ ID65 to SEQ ID69). It is important to ensure the correct reading frame is maintained for the spacer, nociceptin and restriction sequences and that the *XbaI* sequence is not preceded by the bases, TC which would result on DAM methylation. The DNA sequence is screened for restriction sequence incorporation and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector.

The spacers that were created included:

Table 2

<u>Code</u>	Protein sequence of the linker	SEQ ID of the linker-DNA
GS10	ALAGGGGSALVLQ	53
GS15	ALAGGGGSGGGGSALVLQ	54
GS25	ALAGGGGSGGGGSGGGGSGGGGGSALVLQ	55
GS30	ALAGGGGSGGGGSGGGGSGGGGSGGGGGSALVLQ	56
HX27	ALAAEAAAKEAAAKEAAAKAGGGGSALVLQ	57

By way of example, in order to create the LC/A-CPN(GS15)-H_N/A fusion construct (SEQ ID70), the pCR 4 vector encoding the linker (SEQ ID66) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID27) also cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sal*I, *Pst*I, and *Hind*III such as the pMAL vector (NEB). The H_N/A DNA (SEQ ID28) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The final construct contains the LC/A-CPN(GS15)-H_N/A ORF (SEQ ID70) for expression as a protein of the sequence illustrated in SEQ ID71.

15

As a further example, to create the LC/A-CPN(GS25)-H_N/A fusion construct (SEQ ID72), the pCR 4 vector encoding the linker (SEQ ID67) is cleaved with *Bam*HI + *Sal*I restriction enzymes. This cleaved vector then serves as the recipient vector for insertion and ligation of the LC/A DNA (SEQ ID27) cleaved with *Bam*HI + *Sal*I. The resulting plasmid DNA is then cleaved with *Bam*HI + *Hind*III restriction enzymes and the LC/A-linker fragment inserted into a similarly cleaved vector containing a unique multiple cloning site for *Bam*HI, *Sal*I, *Pst*I, and *Hind*III such as the pMAL vector (NEB). The H_N/A DNA (SEQ ID28) is then cleaved with *Pst*I + *Hind*III restriction enzymes and inserted into the similarly cleaved pMAL-LC/A-linker construct. The

20

final construct contains the LC/A-CPN(GS25)-H_N/A ORF (SEQ ID72) for expression as a protein of the sequence illustrated in SEQ ID73.

5 Variants of the LC/A-CPN-H_N/A fusion consisting of GS10, GS30 and HX27 are similarly created. Using the purification methodology described in Example 26, fusion protein is purified from *E. coli* cell paste. Figure 12 illustrates the purified product obtained in the case of LC/A-CPN(GS10)-H_N/A, LC/A-CPN(GS15)-H_N/A, LC/A-CPN(GS25)-H_N/A, LC/A-CPN(GS30)-H_N/A and LC/A-CPN(HX27)-H_N/A.

10 **Example 30 - Assessment of *in vitro* efficacy of an LC/A-nociceptin-H_N/A fusion**

Fusion protein prepared according to Examples 2 and 9 was assessed in the eDRG neuronal cell model.

15 Assays for the inhibition of substance P release and cleavage of SNAP-25 have been previously reported (Duggan *et al.*, 2002, *J. Biol. Chem.*, 277, 34846-34852). Briefly, dorsal root ganglia neurons are harvested from 15-day-old fetal Sprague-Dawley rats and dissociated cells plated onto 24-well plates coated with Matrigel at a density of 1 x 10⁶ cells/well. One day post-plating the cells are treated with 10 μM cytosine β-D-arabinofuranoside for 48 h. Cells are maintained in Dulbecco's minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum, 5 mM L-glutamine, 0.6% D-glucose, 2% B27 supplement, and 100 ng/ml 2.5S mouse nerve growth factor. Cultures are maintained for 2 weeks at 37°C in 95% air/5%
20 CO₂ before addition of test materials.

Release of substance P from eDRG is assessed by enzyme-linked immunosorbent assay. Briefly, eDRG cells are washed twice with low potassium-balanced salt solution (BSS: 5 mM KCl, 137 mM NaCl, 1.2 mM MgCl₂, 5 mM glucose, 0.44 mM
30 KH₂PO₄, 20 mM HEPES, pH 7.4, 2 mM CaCl₂). Basal samples are obtained by incubating each well for 5 min. with 1 ml of low potassium BSS. After removal of this

buffer, the cells are stimulated to release by incubation with 1 ml of high potassium buffer (BSS as above with modification to include 100 mM KCl isotonicly balanced with NaCl) for 5 min. All samples are removed to tubes on ice prior to assay of substance P. Total cell lysates are prepared by addition of 250- μ l of 2 M acetic acid/0.1% trifluoroacetic acid to lyse the cells, centrifugal evaporation, and resuspension in 500 μ l of assay buffer. Diluted samples are assessed for substance P content. Substance P immunoreactivity is measured using Substance P Enzyme Immunoassay Kits (Cayman Chemical Company or R&D Systems) according to manufacturers' instructions. Substance P is expressed in pg/ml relative to a standard substance P curve run in parallel.

SDS-PAGE and Western blot analysis were performed using standard protocols (Novex). SNAP-25 proteins were resolved on a 12% Tris/glycine polyacrylamide gel (Novex) and subsequently transferred to nitrocellulose membrane. The membranes were probed with a monoclonal antibody (SMI-81) that recognises cleaved and intact SNAP-25. Specific binding was visualised using peroxidase-conjugated secondary antibodies and a chemiluminescent detection system. Cleavage of SNAP-25 was quantified by scanning densitometry (Molecular Dynamics Personal SI, ImageQuant data analysis software). Percent SNAP-25 cleavage was calculated according to the formula: $(\text{Cleaved SNAP-25}/(\text{Cleaved}+\text{Intact SNAP-25}))\times 100$.

Following exposure of eDRG neurons to an LC/A-nociceptin-H_N/A fusion (termed CPN-A), both inhibition of substance P release and cleavage of SNAP-25 are observed (Figure 13). After 24 h exposure to the fusion, 50% of maximal SNAP-25 cleavage is achieved by a fusion concentration of 6.3 ± 2.5 nM.

The effect of the fusion is also assessed at defined time points following a 16 h exposure of eDRG to CPN-A. Figure 14 illustrates the prolonged duration of action of the CPN-A fusion protein, with measurable activity still being observed at 28 days post exposure.

Example 31 - Assessment of *in vitro* efficacy of an LC/A-nociceptin variant-H_N/A fusion

5 Fusion protein prepared according to Examples 8 and 9 was assessed in the eDRG neuronal cell mode using the method described in Example 30.

Following exposure of eDRG neurons to an LC/A-nociceptin variant-H_N/A fusion (termed CPNv-A), both inhibition of substance P release and cleavage of SNAP-25 are observed. After 24 h exposure to the fusion, 50% of maximal SNAP-25 cleavage
10 is achieved by a fusion concentration of 1.4±0.4 nM (Figure 15).

The effect of the fusion is also assessed at defined time points following a 16 h exposure of eDRG to CPN-A. Figure 16 illustrates the prolonged duration of action of the CPN-A fusion protein, with measurable activity still being observed at 24 days
15 post exposure.

The binding capability of the CPNv-A fusion protein is also assessed in comparison to the CPN-A fusion. Figure 17 illustrates the results of a competition experiment to determine binding efficacy at the ORL-1 receptor. CPNv-A is demonstrated to
20 displace [3H]-nociceptin, thereby confirming that access to the receptor is possible with the ligand in the central presentation format.

Example 32 - Preparation of an LC/A-nociceptin variant-H_N/A fusion protein that is activated by treatment with Enterokinase

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Following the methods used in Examples 1 and 2, the LC/A (SEQ ID27) and H_N/A (SEQ ID28) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sall*-linker-enterokinase protease site-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID74). The final construct contains
30 the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID75) for expression as a protein of the sequence illustrated in SEQ ID76. The fusion protein is termed CPNv(Ek)-A. Figure 18 illustrates the purification of CPNv(Ek)-A from *E.*

coli following the methods used in Example 26 but using Enterokinase for activation at 0.00064 µg per 100 µg of fusion protein.

Example 33 - Assessment of *in vitro* efficacy of an LC/A-nociceptin variant-H_N/A fusion that has been activated by treatment with enterokinase

The CPNv(Ek)-A prepared in Example 32 is obtained in a purified form and applied to the eDRG cell model to assess cleavage of SNAP-25 (using methodology from Example 30). Figure 19 illustrates the cleavage of SNAP-25 following 24 h exposure of eDRG to CPNv(Ek)-A. The efficiency of cleavage is observed to be similar to that achieved with the Factor Xa-cleaved material, as recorded in Example 31.

Example 34 - Preparation of an LC/C-nociceptin variant-H_N/C fusion protein with a Factor Xa activation linker derived from serotype A

Following the methods used in Example 21, the LC/C (SEQ ID31) and H_N/C (SEQ ID32) are created and inserted into the A serotype nociceptin variant linker arranged as *Bam*HI-*Sall*-linker-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID77). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID78) for expression as a protein of the sequence illustrated in SEQ ID79. The fusion protein is termed CPNv-C (act. A). Figure 20 illustrates the purification of CPNv-C (act. A) from *E. coli* following the methods used in Example 26.

Example 35 - Assessment of *in vitro* efficacy of an LC/C-nociceptin variant-H_N/C fusion protein

Following the methods used in Example 26, the CPNv-C (act. A) prepared in Example 34 is obtained in a purified form and applied to the eDRG cell model to assess cleavage of SNAP-25 (using methodology from Example 30). After 24 h exposure to the fusion, 50% of maximal syntaxin cleavage is achieved by a fusion concentration of 3.1±2.0 nM. Figure 21 illustrates the cleavage of syntaxin following

24 h exposure of eDRG to CPNv-C (act. A).

Example 36 - Assessment of *in vivo* efficacy of an LC/A-nociceptin-HN/A fusion

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The ability of an LC/A-nociceptin- H_N/A fusion (CPN/A) to inhibit acute capsaicin-induced mechanical allodynia is evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals are evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10
10 stimuli x 3 trials) prior to recruitment into the study, after subcutaneous treatment with CPN/A but before capsaicin, and following capsaicin challenge post-injection of CPN/A (average of responses at 15' and 30'). Capsaicin challenge is achieved by injection of 10 µL of a 0.3% solution. Sample dilutions are prepared in 0.5% BSA/saline. Figure 22 illustrates the reversal of mechanical allodynia that is
15 achieved by pre-treatment of the animals with a range of concentrations of LC/A-nociceptin-HN/A fusion.

The ability of an LC/A-nociceptin-HN/A fusion (CPN/A) to inhibit streptozotocin (STZ)-induced mechanical (tactile) allodynia in rats is evaluated. STZ-induced
20 mechanical allodynia in rats is achieved by injection of streptozotocin (i.p. or i.v.) which yields destruction of pancreatic β-cells leading to loss of insulin production, with concomitant metabolic stress (hyperglycemia and hyperlipidemia). As such, STZ induces Type I diabetes. In addition, STZ treatment leads to progressive development of neuropathy, which serves as a model of chronic pain with
25 hyperalgesia and allodynia that may reflect signs observed in diabetic humans (peripheral diabetic neuropathy).

Male Sprague-Dawley rats (250-300 g) are treated with 65 mg/kg STZ in citrate buffer (I.V.) and blood glucose and lipid are measured weekly to define the
30 readiness of the model. Paw Withdrawal Threshold (PWT) is measured in response to a Von Frey filament stimulus series over a period of time. Allodynia is said to be established when the PWT on two consecutive test dates (separated by 1 week)

measures below 6 g on the scale. At this point, rats are randomized to either a saline group (negative efficacy control), gabapentin group (positive efficacy control) or a test group (CPN/A). Test materials (20-25 μ l) are injected subcutaneously as a single injection (except gabapentin) and the PWT is measured at 1 day post-treatment and periodically thereafter over a 2-week period. Gabapentin (30 mg/kg i.p. @ 3 ml/kg injection volume) is injected daily, 2 hours prior to the start of PWT testing. Figure 23 illustrates the reversal of allodynia achieved by pre-treatment of the animals with 750 ng of CPN/A. Data were obtained over a 2-week period after a single injection of CPN/A

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Example 37 - Assessment of *in vivo* efficacy of an LC/A-nociceptin variant-H_N/A fusion

The ability of an LC/A-nociceptin variant-H_N/A fusion (CPNv/A) to inhibit capsaicin-induced mechanical allodynia is evaluated following subcutaneous intraplantar injection in the rat hind paw. Test animals are evaluated for paw withdrawal frequency (PWF%) in response to a 10 g Von Frey filament stimulus series (10 stimuli x 3 trials) prior to recruitment into the study (Pre-Treat); after subcutaneous intraplantar treatment with CPNv/A but before capsaicin (Pre-CAP); and following capsaicin challenge post-injection of CPNv/A (average of responses at 15' and 30'; CAP). Capsaicin challenge is achieved by injection of 10 μ L of a 0.3% solution. Sample dilutions are prepared in 0.5% BSA/saline.

Figure 24 illustrates the reversal of allodynia that is achieved by pre-treatment of the animals with a range of concentrations of LC/A-nociceptin variant-H_N/A fusion in comparison to the reversal achieved with the addition of LC/A-nociceptin-H_N/A fusion. These data are expressed as a normalized paw withdrawal frequency differential, in which the difference between the peak response (post-capsaicin) and the baseline response (pre-capsaicin) is expressed as a percentage. With this analysis, it can be seen that CPNv/A is more potent than CPN/A since a lower dose of CPNv/A is required to achieve similar analgesic effect to that seen with CPN/A.

Example 38 - Preparation of an LC/A-leu enkephalin-H_N/A fusion protein

Due to the small, five-amino acid, size of the leu-enkephalin ligand the LC/A-leu-enkephalin-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID39) as a template. Oligonucleotides are designed encoding the YGGFL leu-enkephalin peptide, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID39) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC-linker-leu enkephalin-spacer-H_N ORF (SEQ ID80) for expression as a protein of the sequence illustrated in SEQ ID81. The fusion protein is termed CPLE-A. Figure 25 illustrates the purification of CPLE-A from *E. coli* following the methods used in Example 26.

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Example 39 – Expression and purification of an LC/A-beta-endorphin-H_N/A fusion protein

Following the methods used in Example 26, and with the LC/A-beta-endorphin-H_N/A fusion protein (termed CPBE-A) created in Example 24, the CPBE-A is purified from *E. coli*. Figure 26 illustrates the purified protein as analysed by SDS-PAGE.

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Example 40 - Preparation of an LC/A-nociceptin mutant-H_N/A fusion protein

Due to the single amino acid modification necessary to mutate the nociceptin sequence at position 1 from a Phe to a Tyr, the LC/A-nociceptin mutant-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin-H_N/A fusion (SEQ ID39) as a template. Oligonucleotides are designed encoding tyrosine at position 1 of the nociceptin sequence, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin-H_N/A fusion (SEQ ID39) either side on the nociceptin

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section. The SDM product is checked by sequencing and the final construct containing the LC/A-nociceptin mutant-spacer-H_N/A fusion ORF (SEQ ID82) for expression as a protein of the sequence illustrated in SEQ ID83. The fusion protein is termed CPOP-A. Figure 27 illustrates the purification of CPOP-A from *E. coli* following the methods used in Example 26.

Example 41 - Preparation and assessment of an LC/A-nociceptin variant mutant-H_N/A fusion protein

Due to the single amino acid modification necessary to mutate the nociceptin sequence at position 1 from a Phe to a Tyr, the LC/A-nociceptin variant mutant-H_N/A fusion is created by site directed mutagenesis [for example using Quickchange (Stratagene Inc.)] using the LC/A-nociceptin variant-H_N/A fusion (SEQ ID51) as a template. Oligonucleotides are designed encoding tyrosine at position 1 of the nociceptin sequence, ensuring standard *E. coli* codon usage is maintained and no additional restriction sites are incorporated, flanked by sequences complimentary to the linker region of the LC/A-nociceptin variant-H_N/A fusion (SEQ ID51) either side on the nociceptin section. The SDM product is checked by sequencing and the final construct containing the LC/A-nociceptin mutant-spacer-H_N/A fusion ORF (SEQ ID84) for expression as a protein of the sequence illustrated in SEQ ID85. The fusion protein is termed CPOPv-A. Figure 28 illustrates the purification of CPOPv-A from *E. coli* following the methods used in Example 26.

Using methodology described in Example 30, CPOPv-A is assessed for its ability to cleave SNAP-25 in the eDRG cell model. Figure 29 illustrates that CPOPv-A is able to cleave SNAP-25 in the eDRG model, achieving cleavage of 50% of the maximal SNAP-25 after exposure of the cells to approximately 5.9 nM fusion for 24 h.

Example 42 - Preparation of an IgA protease-nociceptin variant-H_N/A fusion protein

The IgA protease amino acid sequence was obtained from freely available database

sources such as GenBank (accession number P09790). Information regarding the structure of the *N. Gonorrhoeae* IgA protease gene is available in the literature (Pohlner *et al.*, Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA-protease, *Nature*, 1987, 325(6103), 458-62). Using Backtranslation tool v2.0 (Entelechon), the DNA sequence encoding the IgA protease modified for *E. coli* expression was determined. A *Bam*HI recognition sequence was incorporated at the 5' end and a codon encoding a cysteine amino acid and *Sall* recognition sequence were incorporated at the 3' end of the IgA DNA. The DNA sequence was screened using MapDraw, (DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required for cloning were removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage was assessed Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables. This optimised DNA sequence (SEQ ID86) containing the IgA open reading frame (ORF) is then commercially synthesized.

The IgA (SEQ ID86) is inserted into the LC-linker-nociceptin variant-spacer-H_N ORF (SEQ ID51) using *Bam*HI and *Sall* restriction enzymes to replace the LC with the IgA protease DNA. The final construct contains the IgA-linker-nociceptin variant-spacer-H_N ORF (SEQ ID87) for expression as a protein of the sequence illustrated in SEQ ID88.

Example 43 - Preparation and assessment of a nociceptin targeted endopeptidase fusion protein with a removable histidine purification tag.

DNA was prepared that encoded a Factor Xa removable his-tag (his6), although it is clear that alternative proteases site such as Enterokinase and alternative purification tags such as longer histidine tags are also possible. Using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)], the DNA sequence encoding the Factor Xa removable his-tag region is determined.

Restriction sites are then incorporated into the DNA sequence and can be arranged as *NheI*-linker-*SpeI*-*PstI*-*H_N/A*-*XbaI*-LEIEGRSGHHHHHHStop codon-*HindIII* (SEQ ID89). The DNA sequence is screened for restriction sequence incorporated and any additional sequences are removed manually from the remaining sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector. In order to create CPNv-A-FXa-HT (SEQ ID90, removable his-tag construct) the pCR 4 vector encoding the removable his-tag is cleaved with *NheI* and *HindIII*. The *NheI* - *HindIII* fragment is then inserted into the LC/A-CPNv-*H_N/A* vector (SEQ ID51) that has also been cleaved by *NheI* and *HindIII*. The final construct contains the LC/A-linker-nociceptin variant-spacer-*H_N*-FXa-Histag-*HindIII* ORF sequences (SEQ ID90) for expression as a protein of the sequence illustrated in SEQ ID91. Figure 30 illustrates the purification of CPNv-A-FXa-HT from *E. coli* following the methods used in Example 26.

Example 44 - Preparation of a leu-enkephalin targeted endopeptidase fusion protein containing a translocation domain derived from diphtheria toxin

The DNA sequence is designed by back translation of the amino acid sequence of the translocation domain of the diphtheria toxin (obtained from freely available database sources such as GenBank (accession number 1XDTT) using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. Restriction sites are then incorporated into the DNA sequence and can be arranged as *NheI*-Linker-*SpeI*-*PstI*- diphtheria translocation domain-*XbaI*-stop codon-*HindIII* (SEQ ID92). *PstI/XbaI* recognition sequences are incorporated at the 5' and 3' ends of the translocation domain respectively of the sequence maintaining the correct reading frame. The DNA sequence is screened (using software such as MapDraw,

DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E. coli* codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence containing the diphtheria translocation domain is then commercially synthesized as *NheI*-Linker-*SpeI*-*PstI*-diphtheria translocation domain-*XbaI*-stop codon-*HindIII* (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector (Invitrogen). The pCR 4 vector encoding the diphtheria translocation domain is cleaved with *NheI* and *XbaI*. The *NheI* – *XbaI* fragment is then inserted into the LC/A-CPLE-H_N/A vector (SEQ ID80) that has also been cleaved by *NheI* and *XbaI*. The final construct contains the LC/A-leu-enkephalin-spacer-diphtheria translocation domain ORF sequences (SEQ ID93) for expression as a protein of the sequence illustrated in SEQ ID94.

Example 45 - Preparation of a nociceptin variant targeted endopeptidase fusion protein containing a LC domain derived from tetanus toxin.

The DNA sequence is designed by back translation of the tetanus toxin LC amino acid sequence (obtained from freely available database sources such as GenBank (accession number X04436) using one of a variety of reverse translation software tools [for example EditSeq best *E. coli* reverse translation (DNASTAR Inc.), or Backtranslation tool v2.0 (Entelechon)]. *BamHI*/*SaI* recognition sequences are incorporated at the 5' and 3' ends respectively of the sequence maintaining the correct reading frame (SEQ ID95). The DNA sequence is screened (using software such as MapDraw, DNASTAR Inc.) for restriction enzyme cleavage sequences incorporated during the back translation. Any cleavage sequences that are found to be common to those required by the cloning system are removed manually from the proposed coding sequence ensuring common *E. coli* codon usage is maintained. *E.*

coli codon usage is assessed by reference to software programs such as Graphical Codon Usage Analyser (Geneart), and the %GC content and codon usage ratio assessed by reference to published codon usage tables (for example GenBank Release 143, 13 September 2004). This optimised DNA sequence containing the tetanus toxin LC open reading frame (ORF) is then commercially synthesized (for example by Entelechon, Geneart or Sigma-Genosys) and is provided in the pCR 4 vector (Invitrogen). The pCR 4 vector encoding the TeNT LC is cleaved with *Bam*HI and *Sal*I. The *Bam*HI – *Sal*I fragment is then inserted into the LC/A-CPNv-H_N/A vector (SEQ ID51) that has also been cleaved by *Bam*HI and *Sal*I. The final construct contains the TeNT LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID96) for expression as a protein of the sequence illustrated in SEQ ID97.

Example 46 - Preparation of an LC/C-nociceptin variant-H_N/C fusion protein with a native serotype C linker that is susceptible to Factor Xa cleavage

Following the methods used in Example 21, the LC/C (SEQ ID31) and H_N/C (SEQ ID32) are created and inserted into the C serotype nociceptin variant linker arranged as *Bam*HI-*Sal*I-linker-nociceptin variant-*Nhe*I-spacer-*Spe*I-*Pst*I-*Xba*I-stop codon-*Hind*III (SEQ ID98). The final construct contains the LC-linker-nociceptin variant-spacer-H_N ORF sequences (SEQ ID99) for expression as a protein of the sequence illustrated in SEQ ID100. The fusion protein is termed CPNv-C (act. C).

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

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NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

Claims:

1. A non-cytotoxic protein conjugate for inhibition or reduction of exocytic fusion in a nociceptive sensory afferent cell, comprising:

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(i) Targeting Moiety (TM),

wherein said TM is an agonist of a receptor present on said nociceptive sensory afferent cell, and wherein said receptor undergoes endocytosis to be incorporated into an endosome within the nociceptive sensory afferent cell;

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(ii) a non-cytotoxic protease or a fragment thereof,

wherein the protease or protease fragment is capable of cleaving a protein of the exocytic fusion apparatus of said nociceptive sensory afferent cell; and

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(iii) a Translocation Domain,

wherein the Translocation Domain translocates the protease or protease fragment from within the endosome, across the endosomal membrane, and into the cytosol of the nociceptive sensory afferent cell.

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2. The non-cytotoxic conjugate of Claim 1, wherein the receptor is an ORL₁ receptor.

3. The non-cytotoxic conjugate of Claim 1 or 2, wherein the TM has at least 70% or at least 80 % homology to SEQ ID No. 2 or a fragment thereof.

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4. The non-cytotoxic conjugate of Claim 1 or 2, wherein the TM has at least 90% homology to SEQ ID No. 2 or a fragment thereof.
- ~~5. The non-cytotoxic conjugate of Claim 1 or Claim 2, wherein the TM has at least 95% homology to SEQ ID No. 2 or a fragment thereof.~~
6. The non-cytotoxic conjugate of Claim 1 or 2, wherein the TM is SEQ ID No. 2 or a fragment thereof.
7. The non-cytotoxic conjugate of Claim 1 or 2, wherein the TM is nociceptin.
8. The non-cytotoxic conjugate of Claim 1 or 2, wherein the TM is selected from the group consisting of SEQ ID Nos. 4, 6, 8, 10, 12, 14.
9. The non-cytotoxic conjugate of any preceding claim, wherein the non-cytotoxic protease is a bacterial protein, or a fragment thereof, capable of cleaving a protein of the exocytic fusion apparatus of the nociceptive sensory afferent cell.
10. The non-cytotoxic conjugate of Claim 9, wherein the non-cytotoxic protease is selected from a clostridial neurotoxin, or an IgA protease.
11. The non-cytotoxic conjugate of any preceding claim, wherein the Translocation Domain is derived from a clostridial source.
12. The non-cytotoxic conjugate of Claim 11, wherein the Translocation Domain is a botulinum H_N domain.
13. The non-cytotoxic conjugate of any preceding claim, wherein the nociceptive sensory afferent cell is a primary nociceptive sensory afferent cell.

14. The non-cytotoxic conjugate according to Claim 1, wherein said conjugate comprises an amino acid sequence selected from the group consisting of SEQ ID NOs 14, 16, 18, 20, 22, 24, and 26.

5 15. A non-cytotoxic conjugate for inhibition or reduction of exocytotic fusion in a nociceptive sensory afferent cell, comprising:

(i) a Targeting Moiety (TM),

10 wherein said TM is an agonist of a receptor present on said nociceptive sensory afferent cell, and wherein said receptor undergoes endocytosis to be incorporated into an endosome within the nociceptive sensory afferent cell;

15 (ii) a DNA sequence encoding a non-cytotoxic protease or a fragment thereof,

20 wherein the DNA sequence is expressible in the nociceptive sensory afferent cell and when so expressed provides a protease or protease fragment capable of cleaving a protein of the exocytic fusion apparatus of said nociceptive sensory afferent cell; and

(iii) a Translocation Domain,

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wherein the Translocation Domain translocates the DNA sequence encoding the protease or protease fragment from within the endosome, across the endosomal membrane, and into the nociceptive sensory afferent cell.

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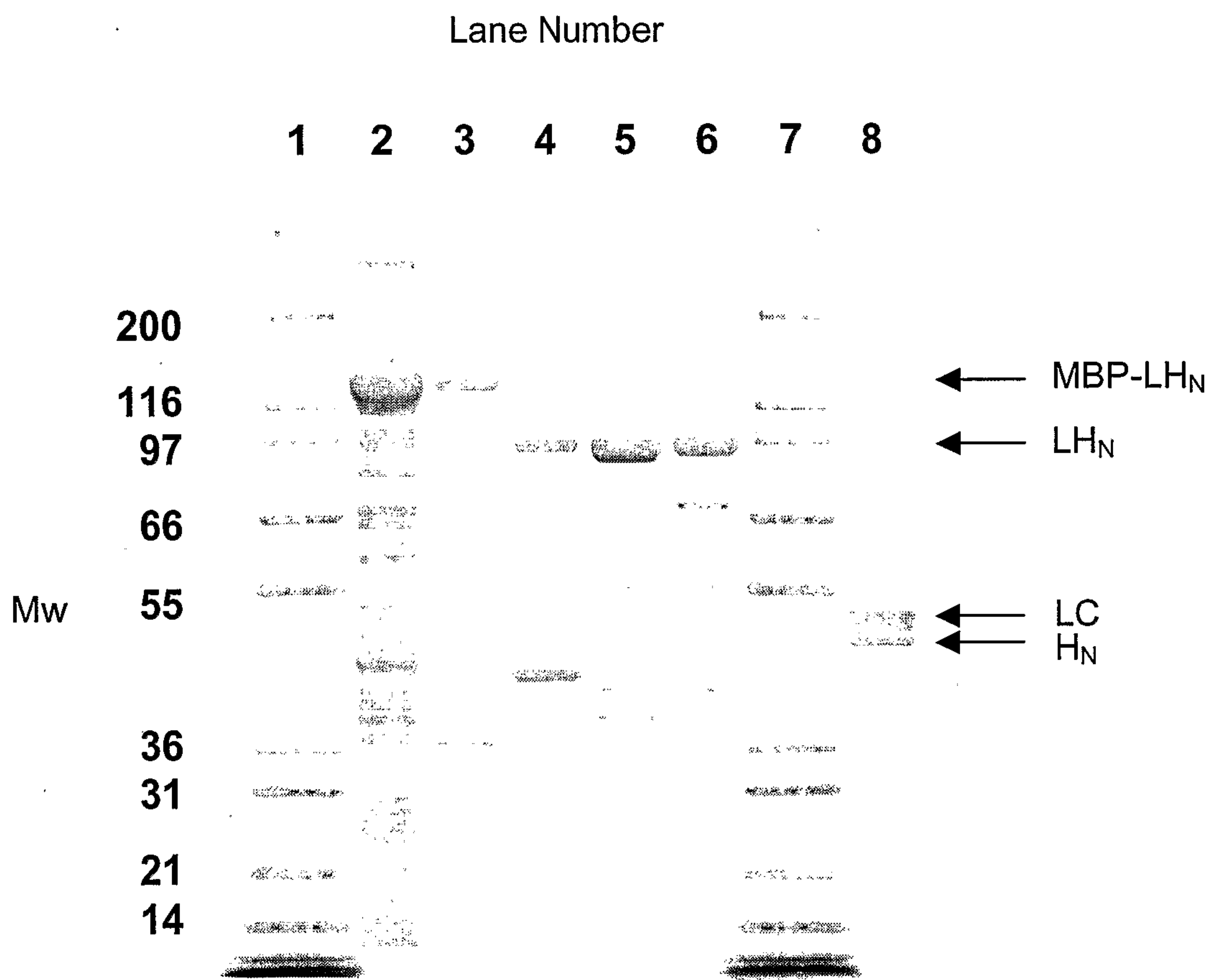
16. The non-cytotoxic conjugate of Claim 15, wherein the receptor is an ORL₁ receptor.

17. The non-cytotoxic conjugate of Claim 15 or 16, wherein the TM has at least 70% or at least 80% homology to SEQ ID No. 2 or a fragment thereof.
- 5 18. The non-cytotoxic conjugate of Claim 15 or 16, wherein the TM has at least 90% homology to SEQ ID No. 2 or a fragment thereof.
19. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the TM has at least 95% homology to SEQ ID No. 2 or a fragment thereof.
- 10 20. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the TM is SEQ ID No. 2 or a fragment thereof.
21. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the TM is nociceptin.
- 15 22. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the TM is selected from the group consisting of SEQ ID Nos. 4, 6, 8, 10, 12, 14.
- 20 23. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the non-cytotoxic protease is a bacterial protein, or a fragment thereof, capable of cleaving a protein of the exocytic fusion apparatus of the nociceptive sensory afferent cell.
- 25 24. The non-cytotoxic conjugate of Claim 23, wherein the non-cytotoxic protease is selected from a clostridial neurotoxin, or an IgA protease.
25. The non-cytotoxic conjugate of Claim 15 or Claim 16, wherein the Translocation Domain is derived from a clostridial source.
- 30 26. The non-cytotoxic conjugate of Claim 25, wherein the Translocation Domain is a botulinum H_N domain.

27. The non-cytotoxic conjugate of Claim 15 or 16, wherein the nociceptive sensory afferent cell is a primary nociceptive sensory afferent cell.
- 5 28. The non-cytotoxic conjugate of any of Claims 1 to 14, wherein the TM, the Translocation Domain and the protease or fragment thereof are covalently linked.
- 10 29. The non-cytotoxic conjugate of any of Claims 15 to 27, wherein the TM, the Translocation Domain and the DNA sequence encoding the protease or fragment thereof, are covalently linked.
- 15 30. A pharmaceutical composition, comprising a conjugate according to any of Claims 1 to 29 and a pharmaceutically acceptable carrier.
31. A DNA construct encoding the conjugate of any of Claims 1 to 14.
32. A DNA construct according to Claim 31, wherein the construct comprises a DNA sequence selected from SEQ ID NOs 13, 15, 17, 19, 21, 23, and 25.
- 20 33. A method of preparing the conjugate of any of Claims 1 to 14, comprising expressing the DNA construct of Claim 30 in a host cell.
34. A method for treating pain, comprising administering to a patient a conjugate according to any of Claim 1-29 or a composition according to Claim 30.
- 25 35. Use of a conjugate according to any of Claims 1-29 or a composition according to Claim 30, for the manufacture of a medicament for treating pain.

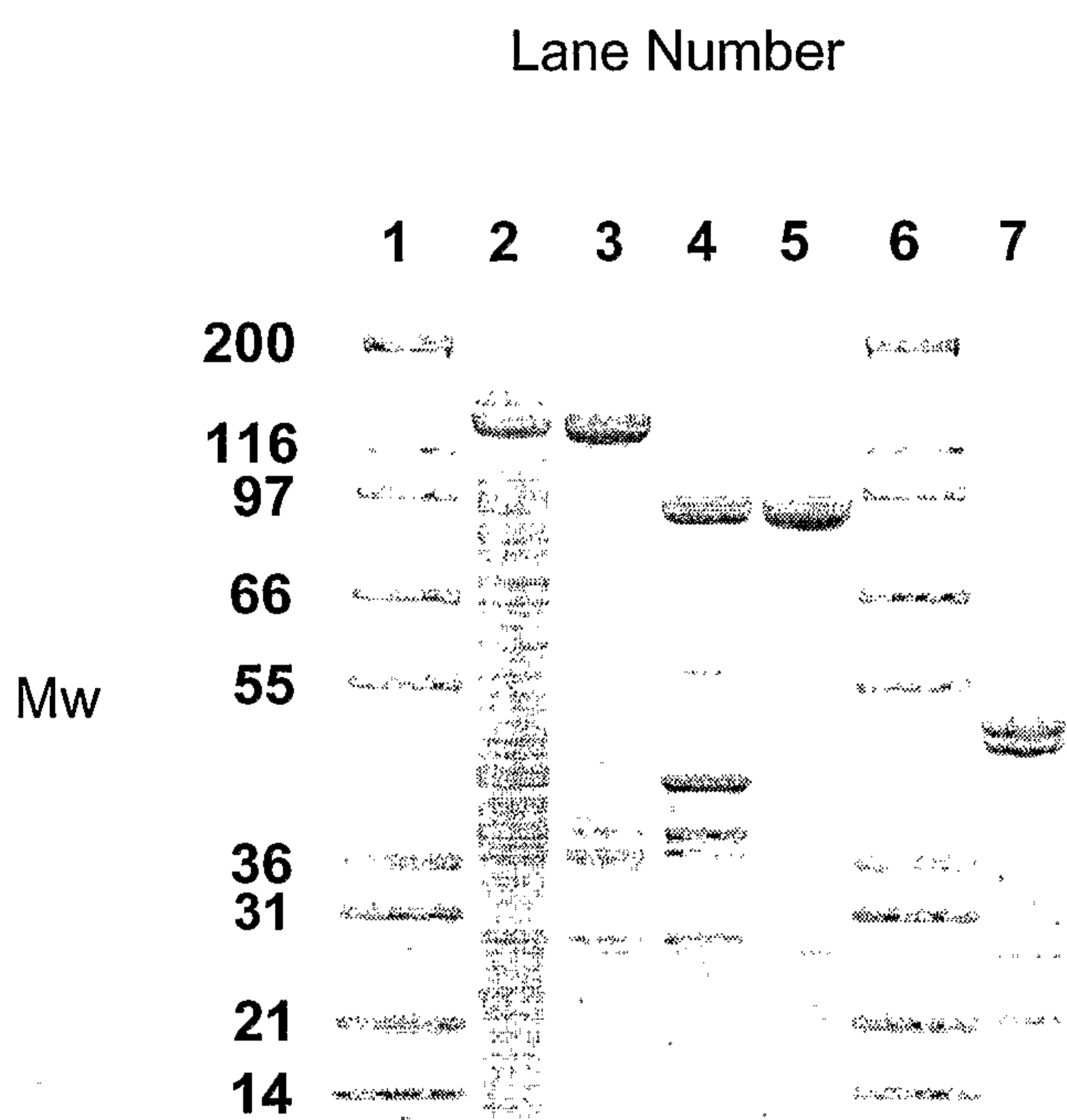
1/32

Figure 1



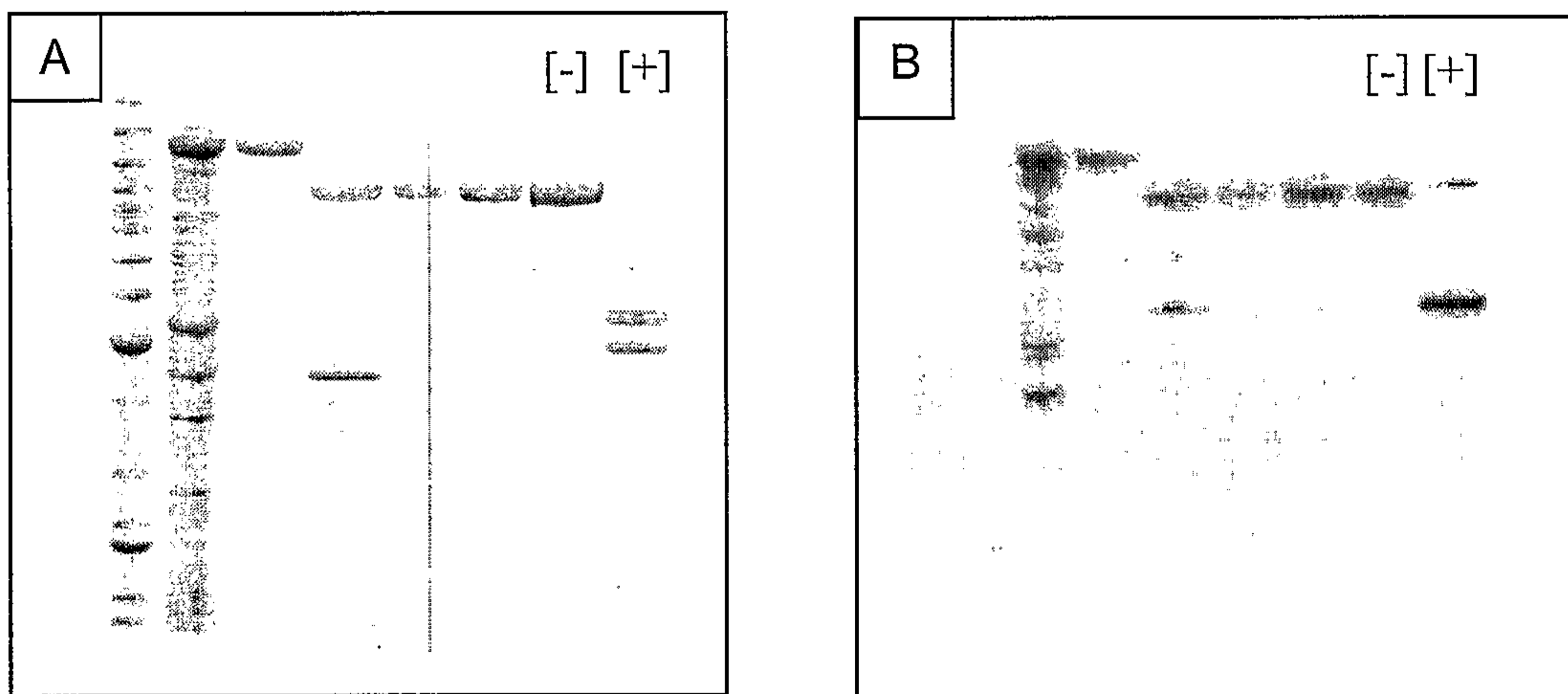
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Figure 2



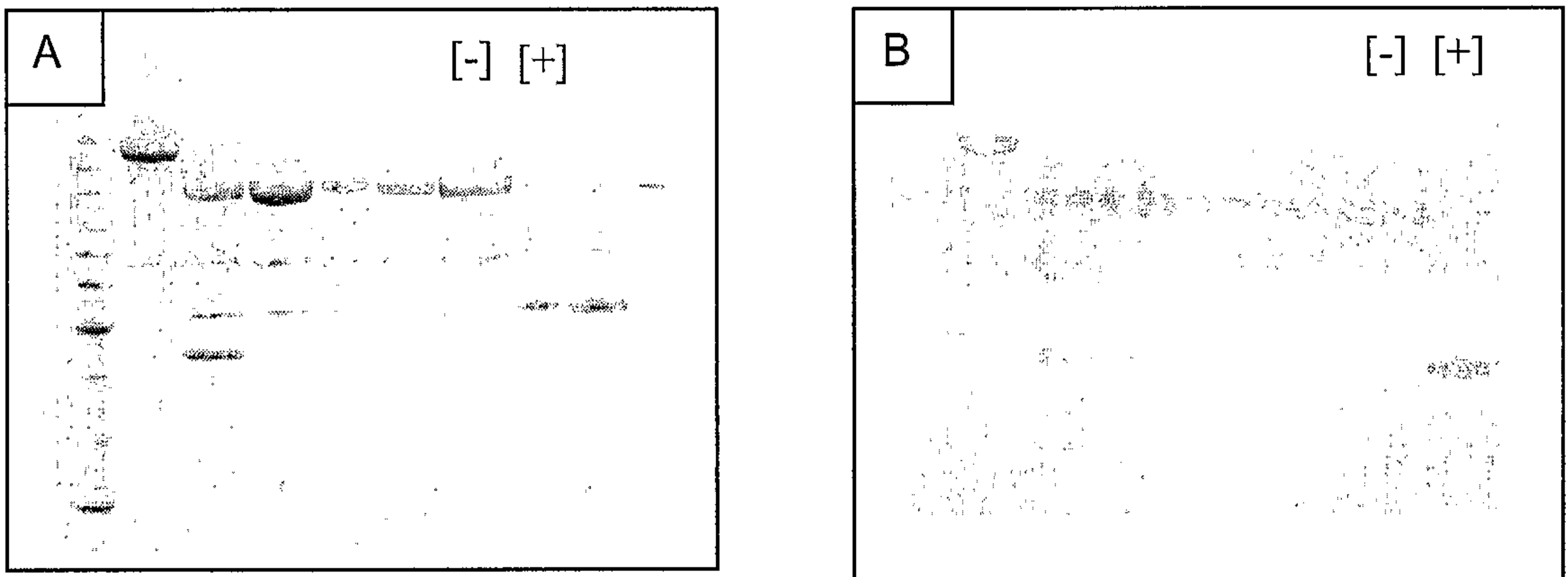
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Figure 3



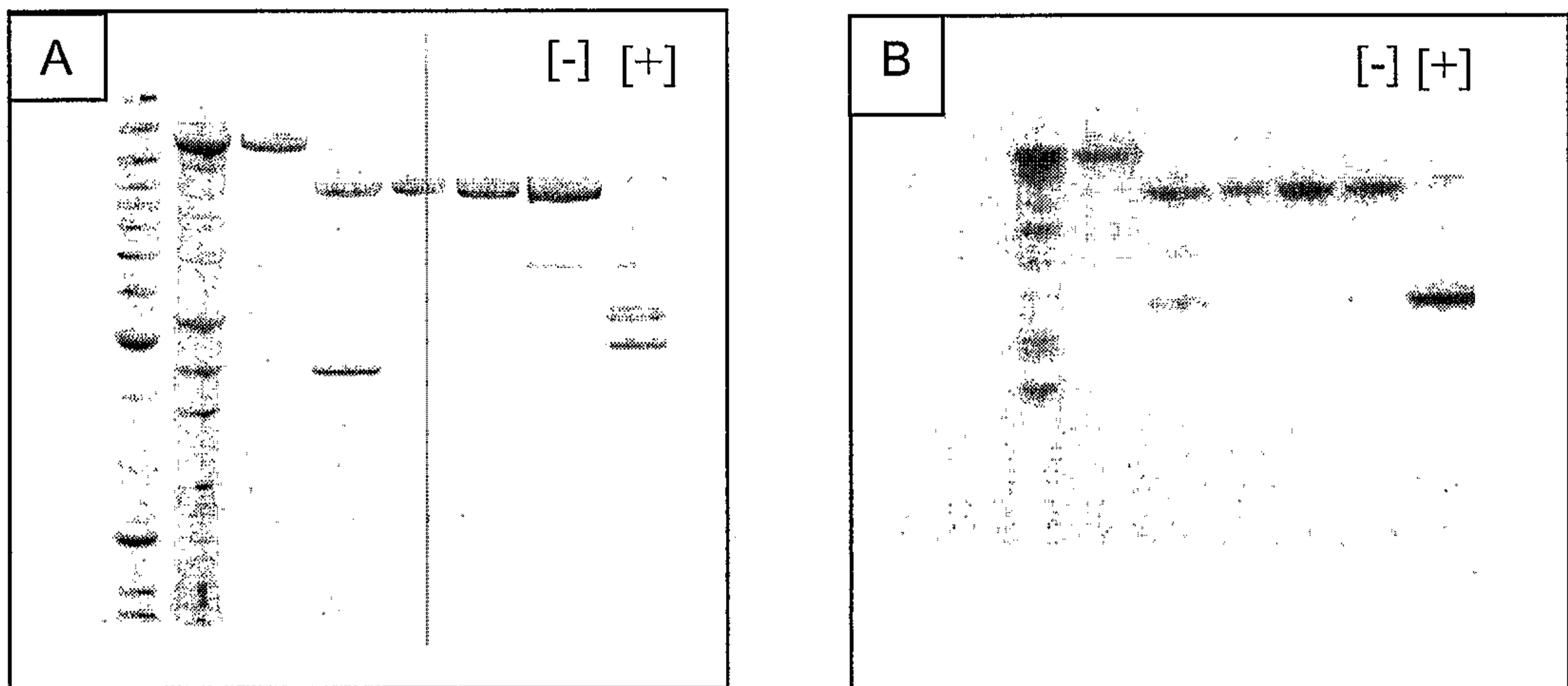
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Figure 4



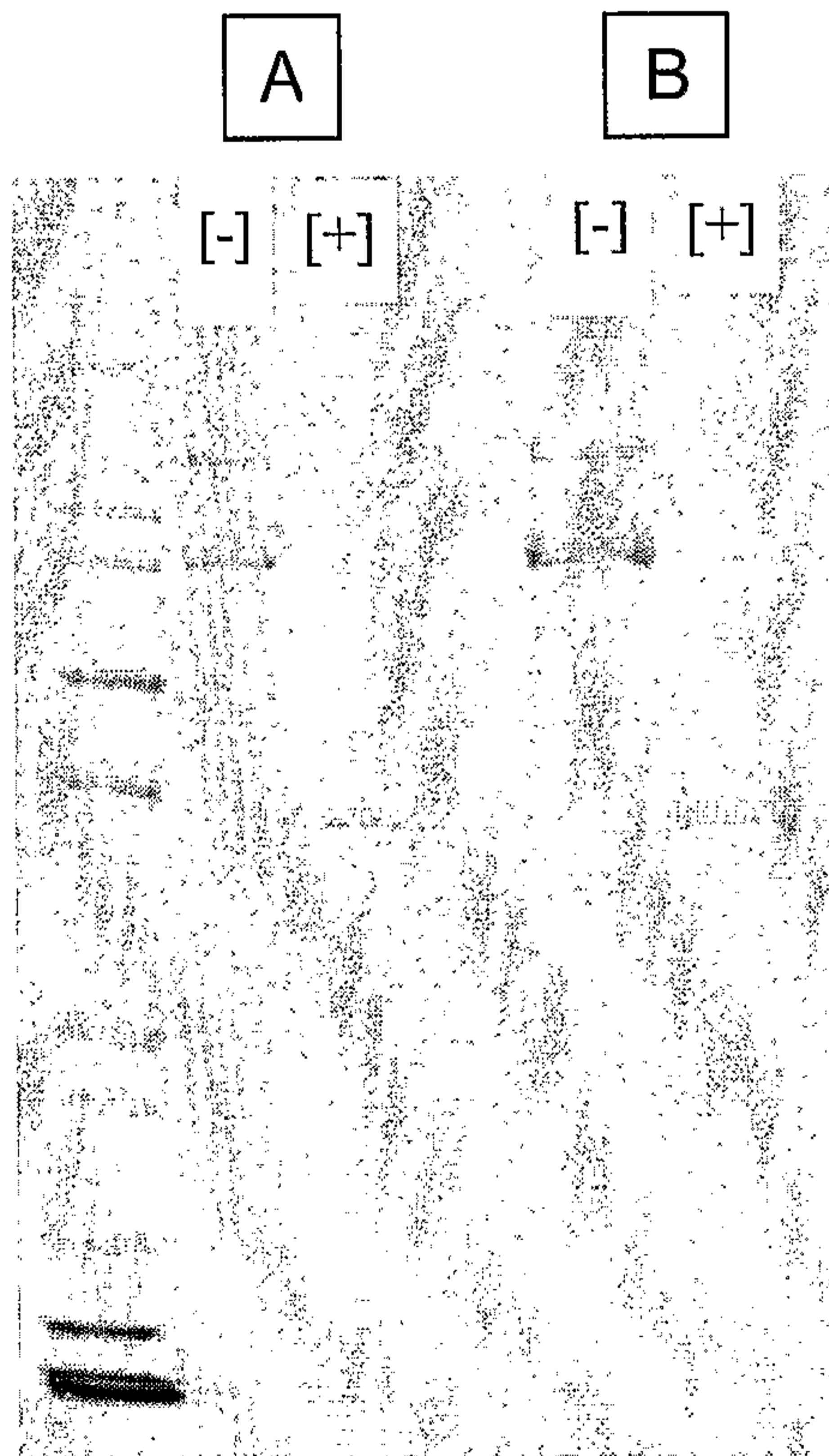
5/32

Figure 5



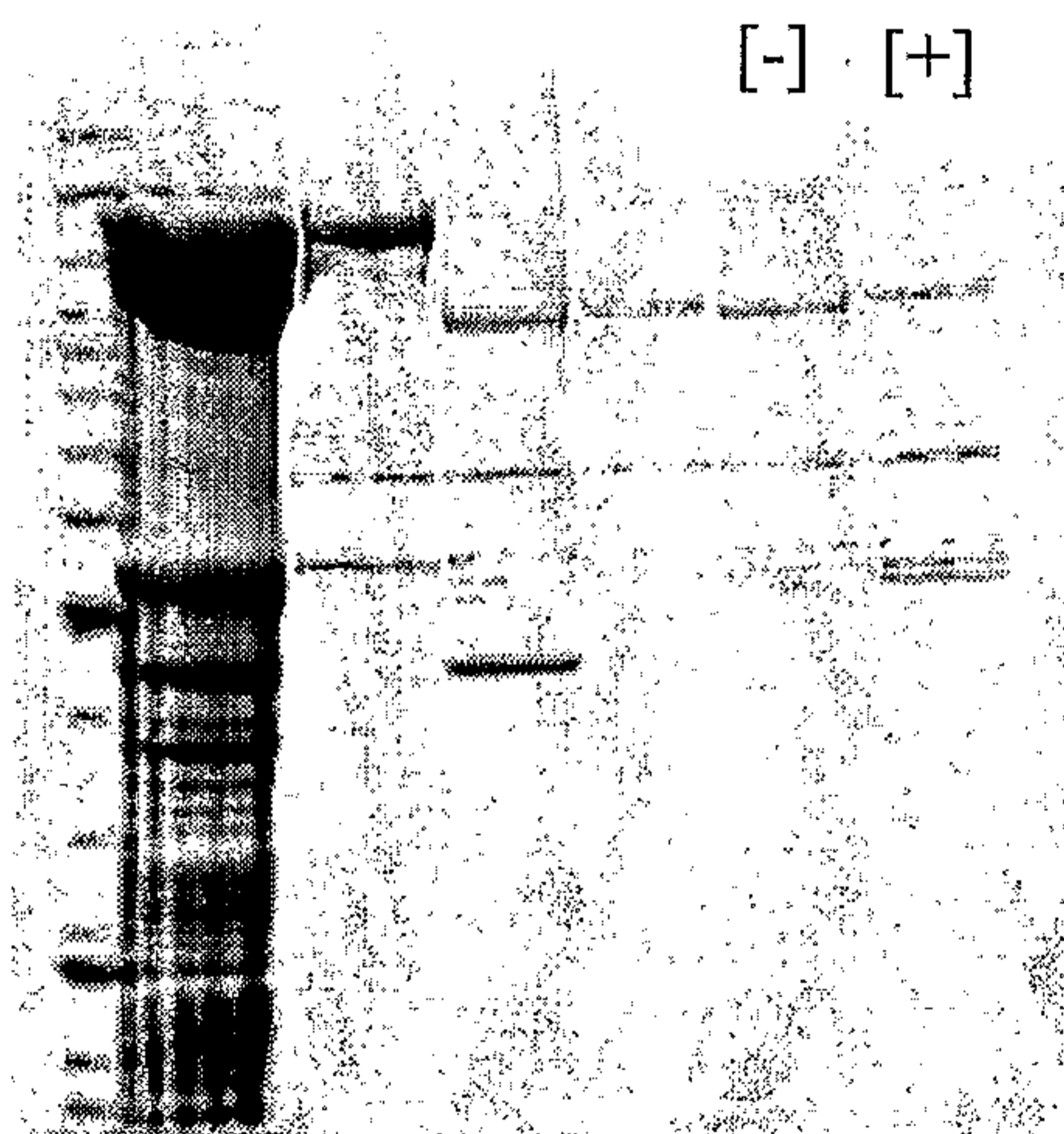
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Figure 6



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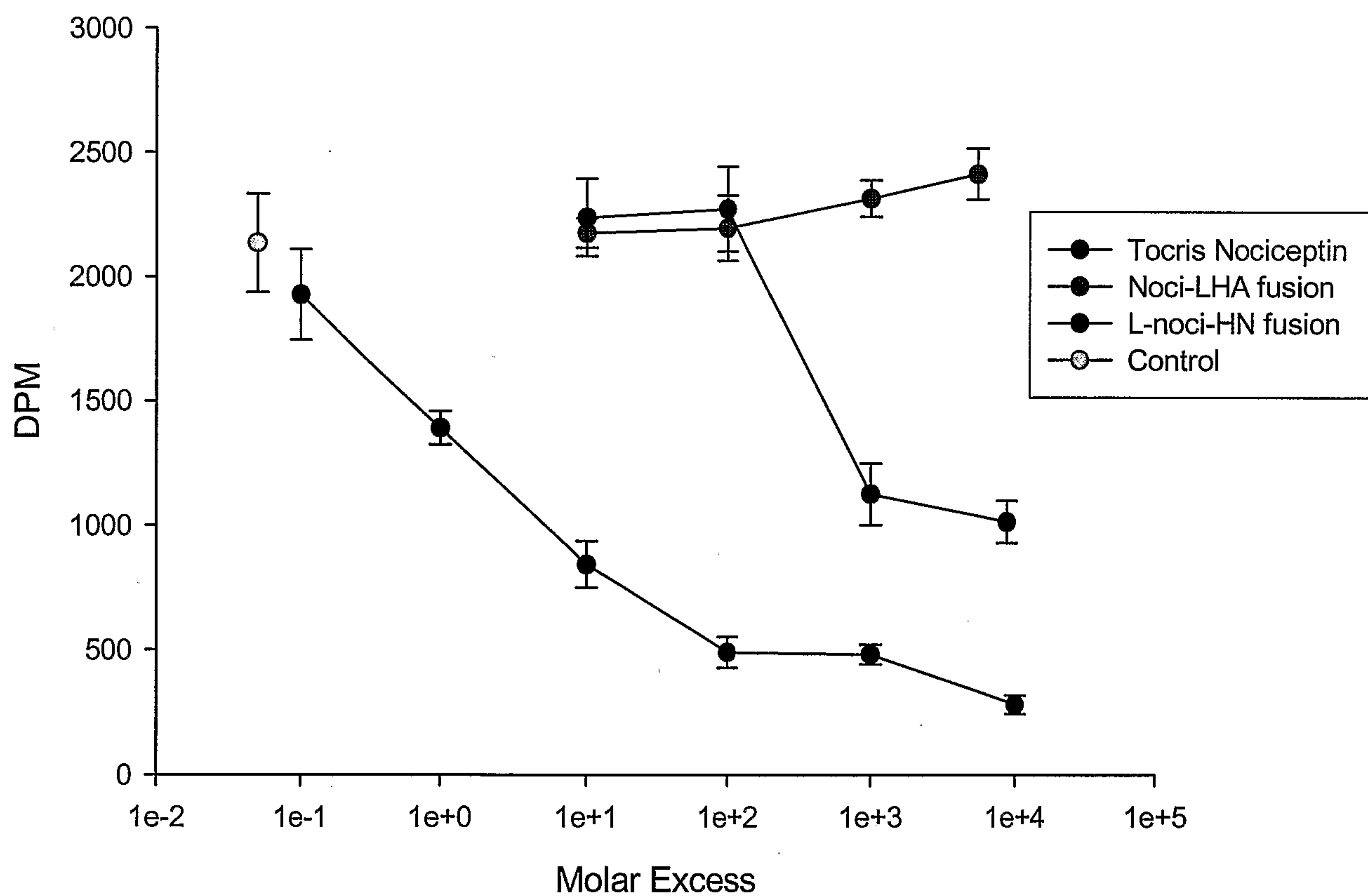
Figure 7



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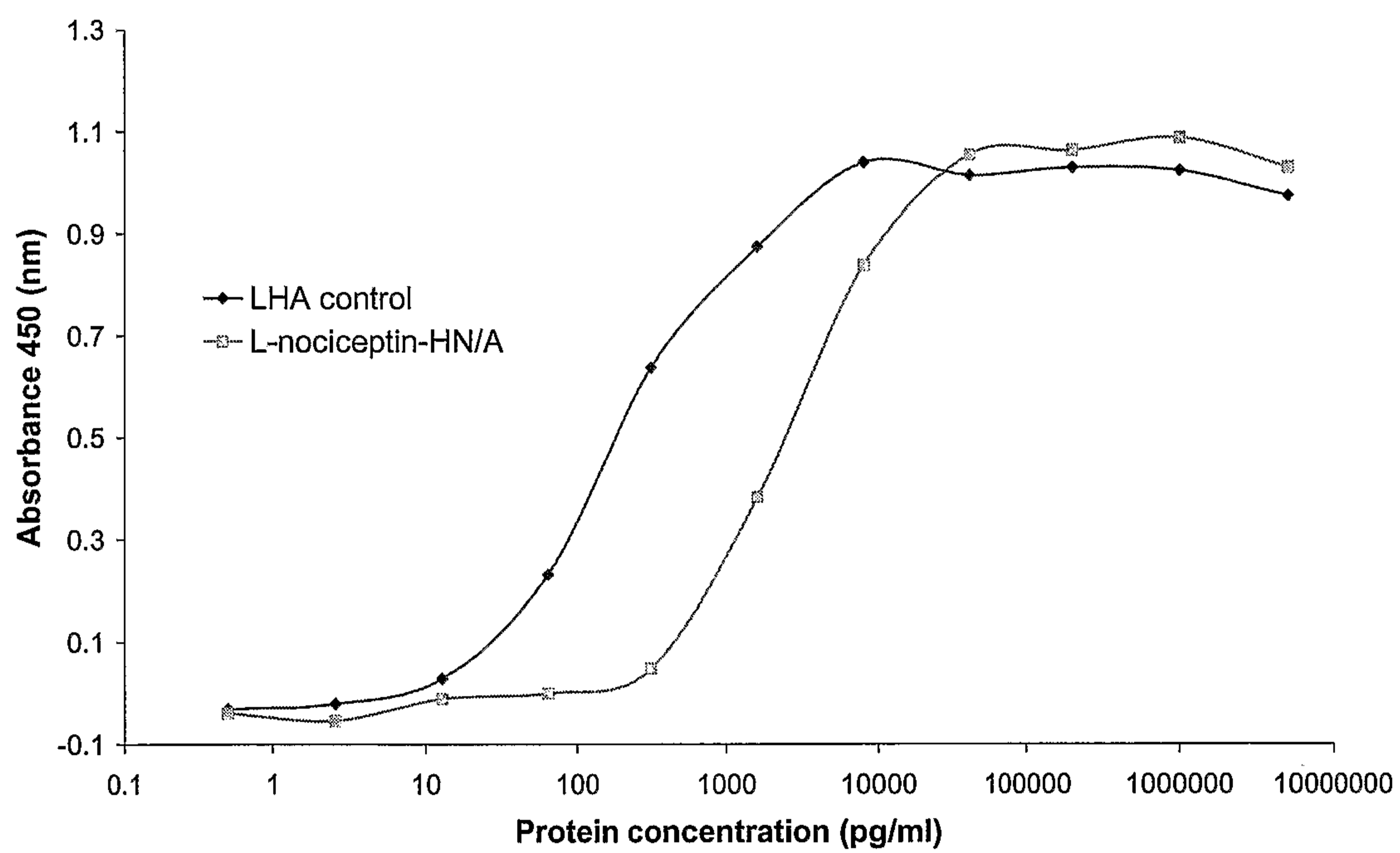
Figure 8

Competition Assay : Nociceptin-LH_N/A Fusions
vs 1nM [³H]-Nociceptin on eDRGs (4°C)



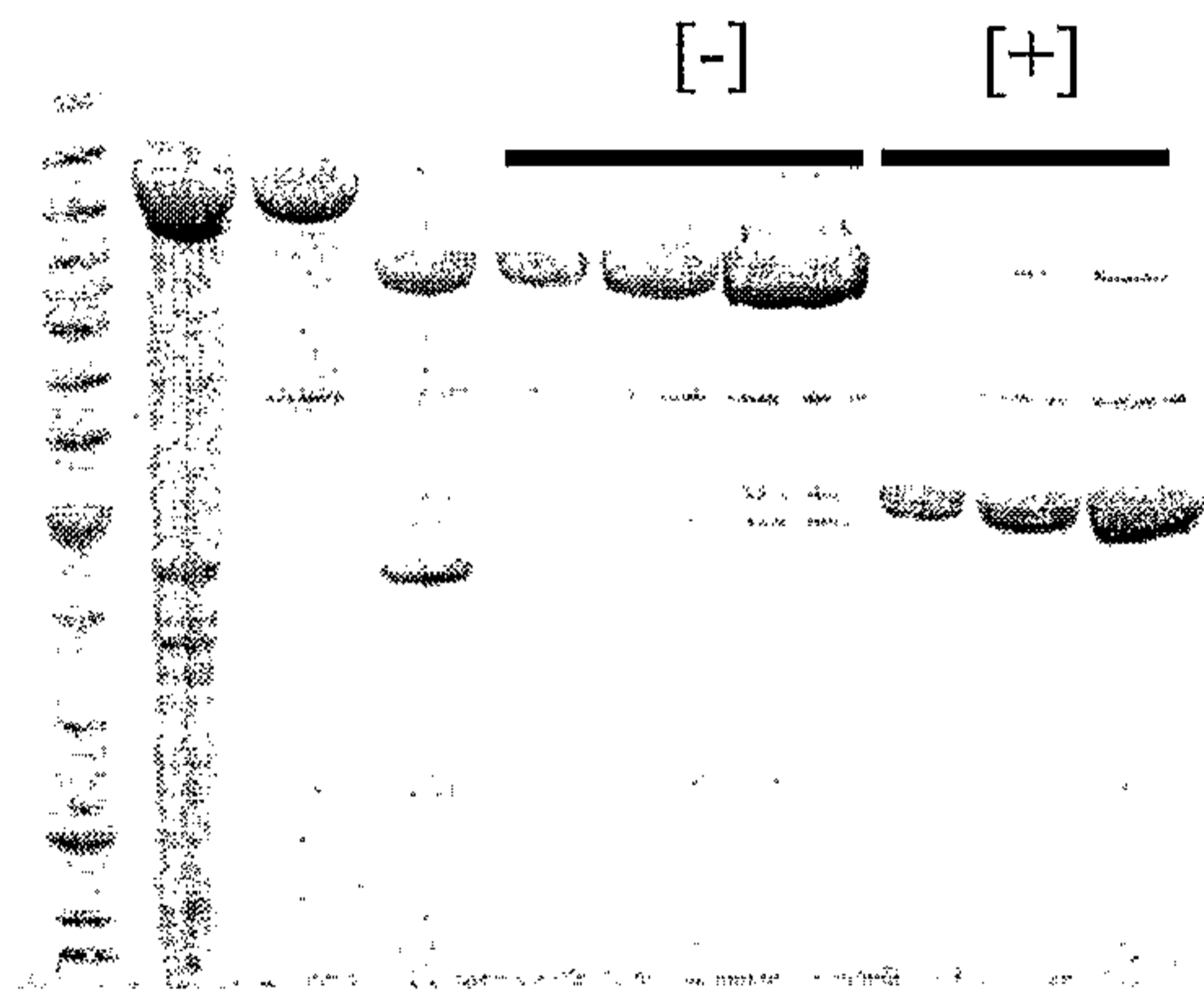
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Figure 9



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Figure 10



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Figure 11

Competition Assay: CPN fusions vs 1nM [3H] - Nociceptin
on eDRGs for 1 hour at 4°C

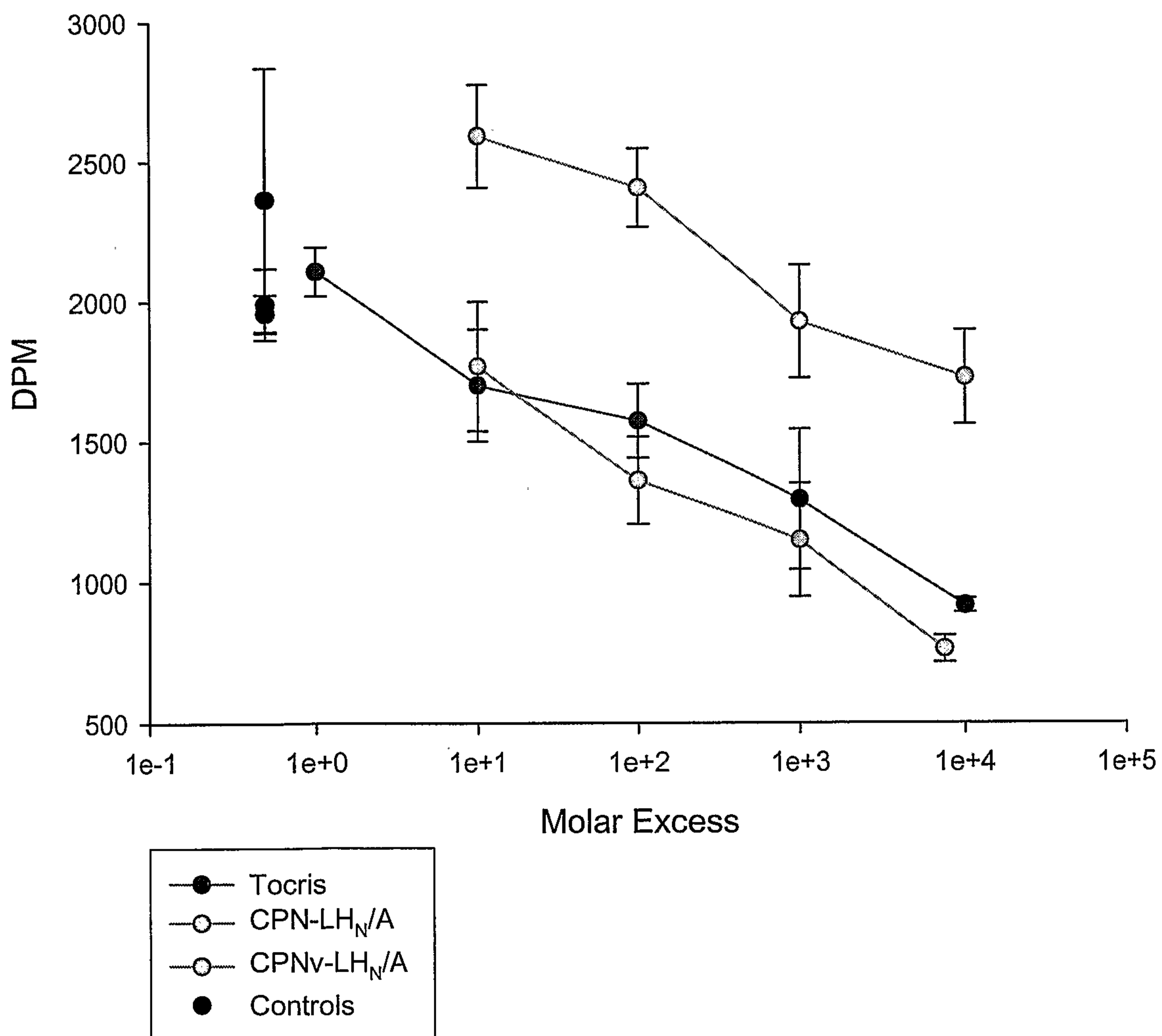
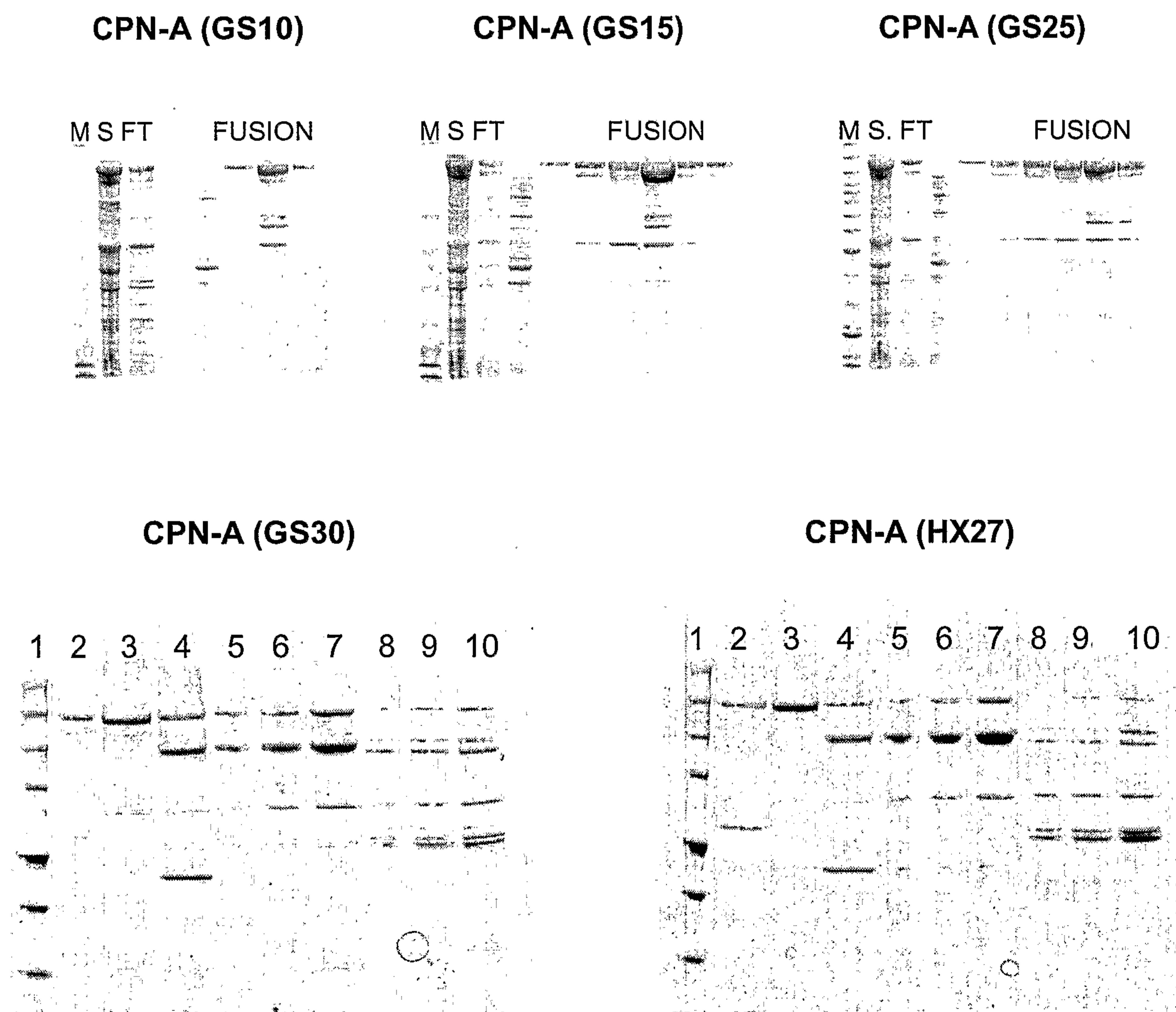


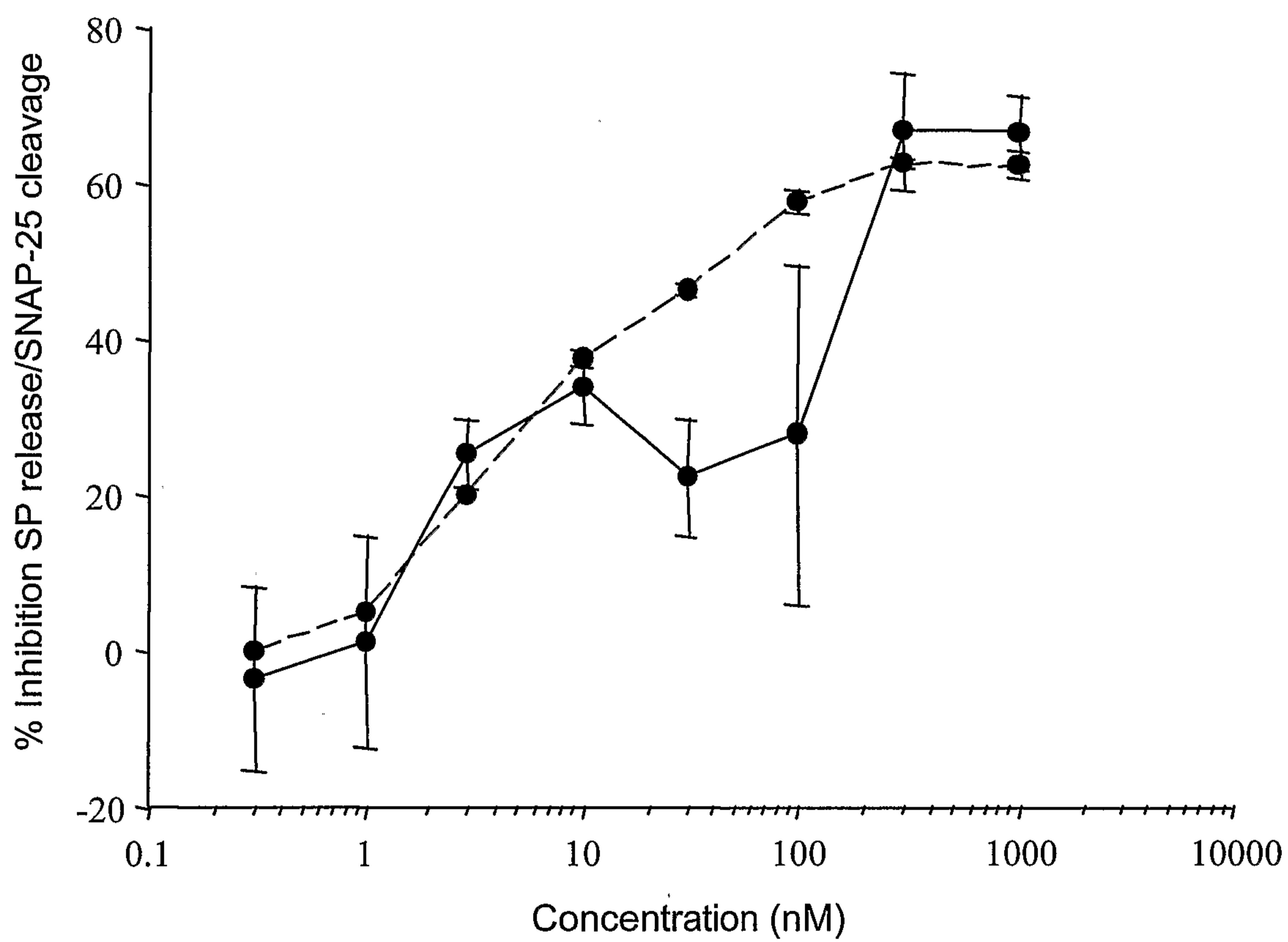
Figure 12



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Figure 13

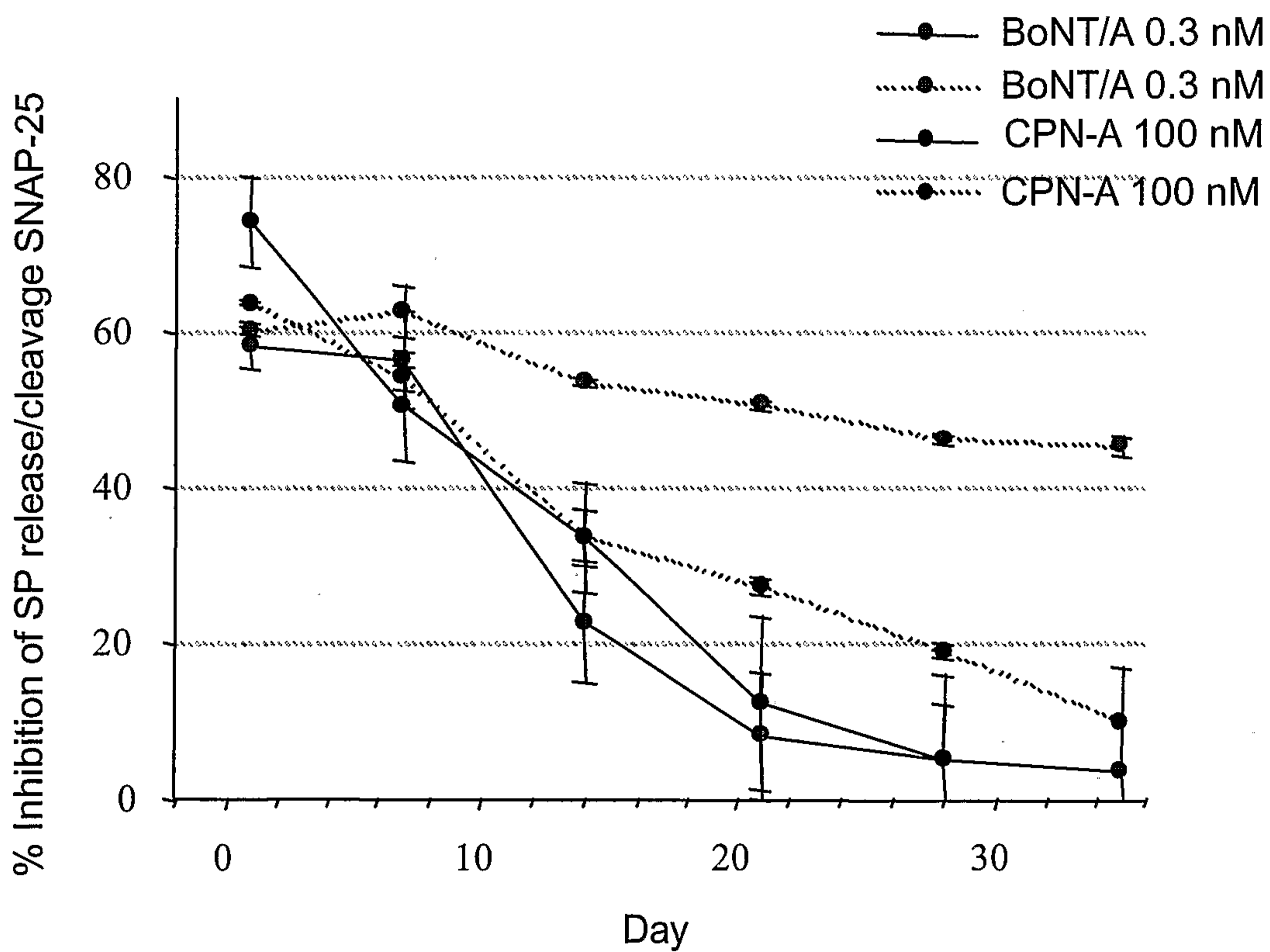
CPN-A on eDRG for 1 Day



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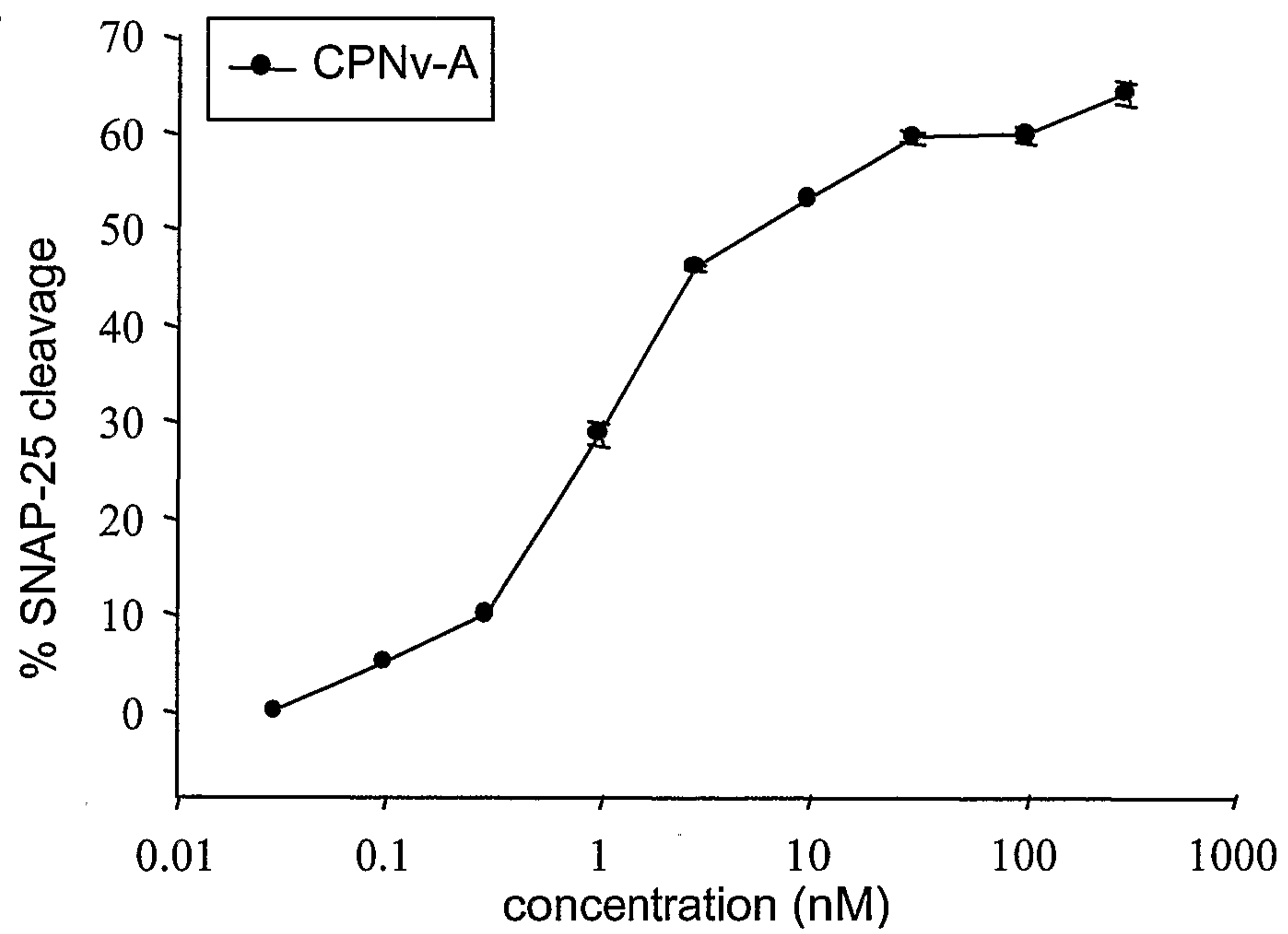
Figure 14

Duration of action following eDRG exposure for 1 Day



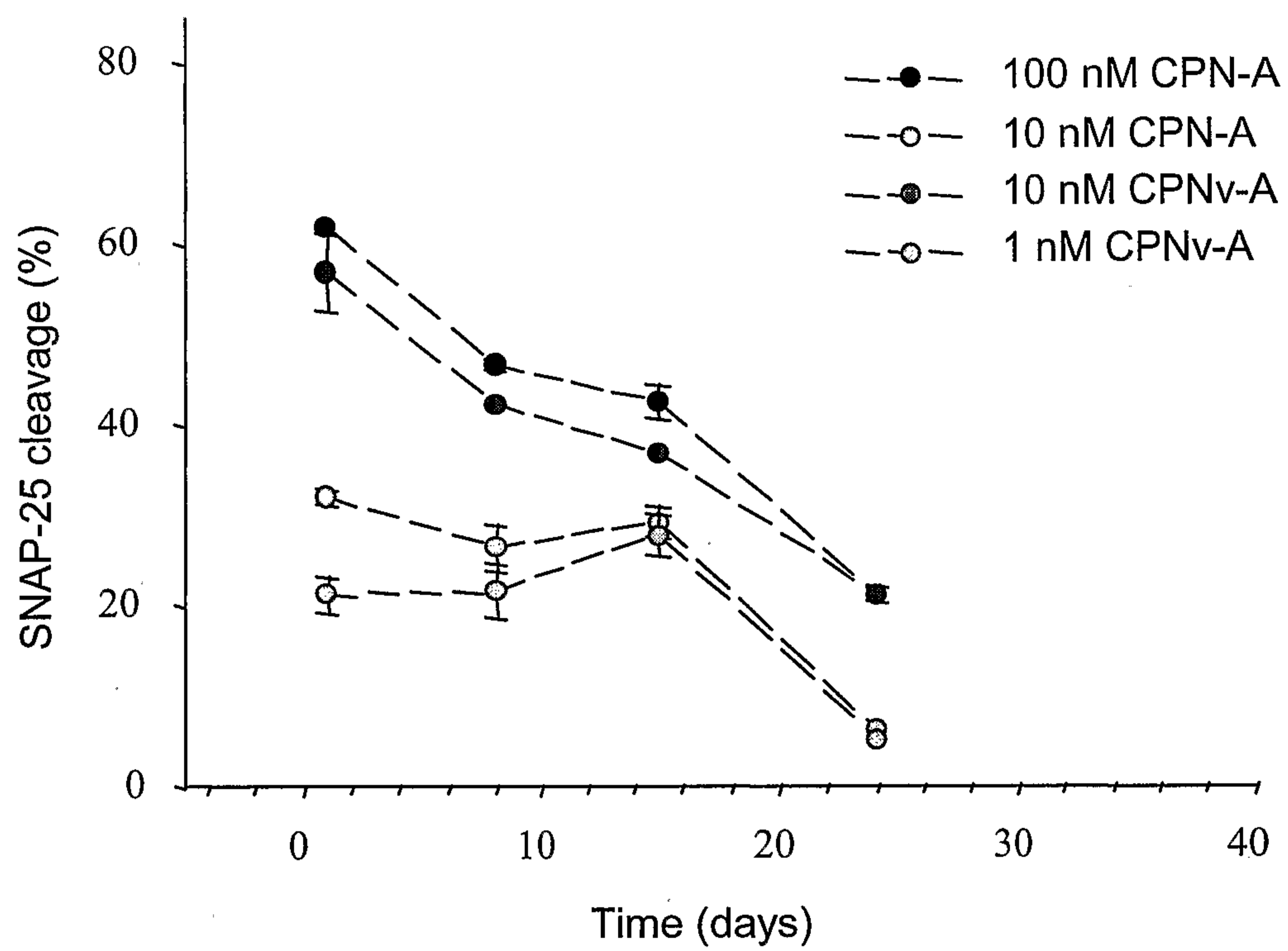
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Figure 15



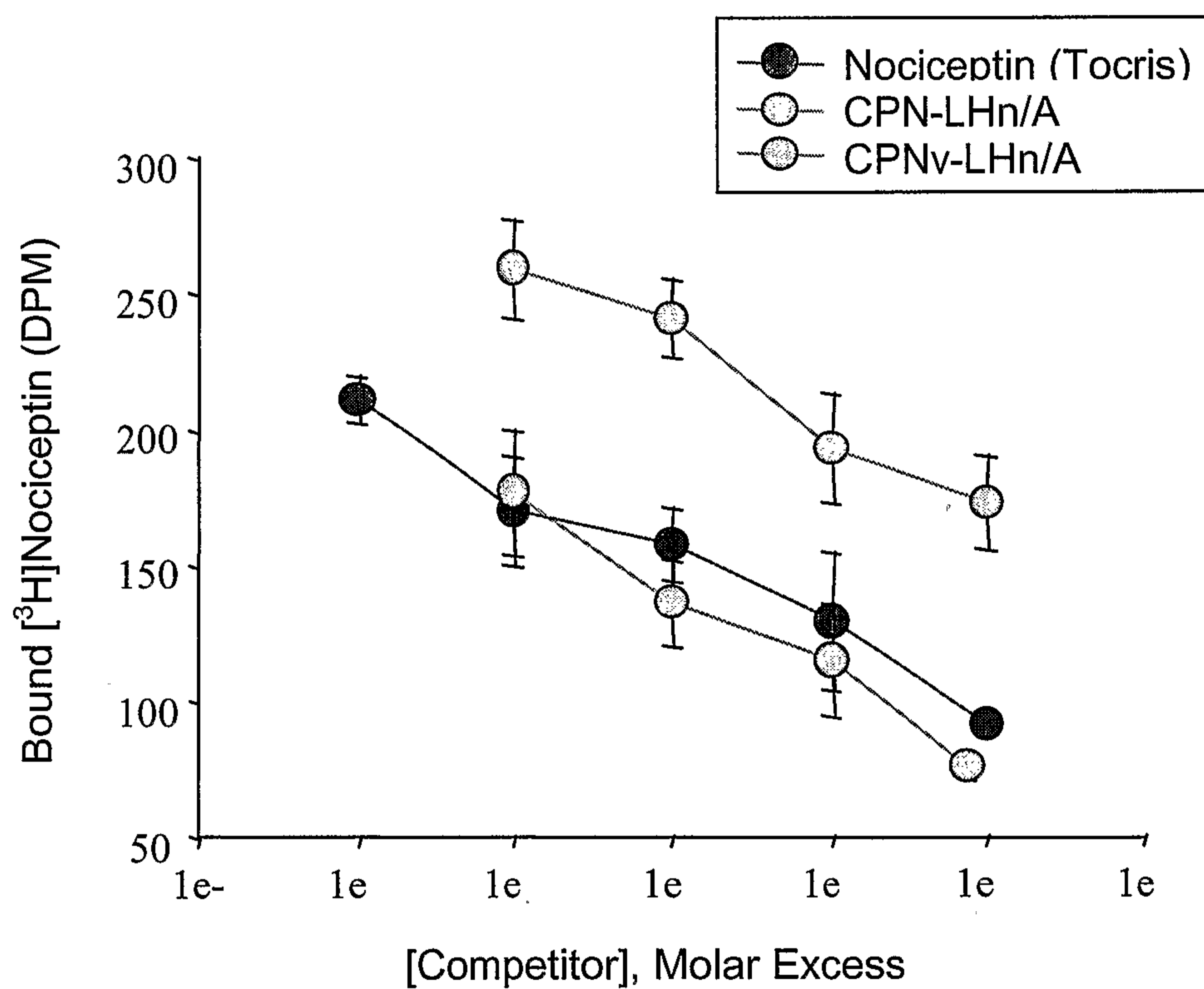
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Figure 16



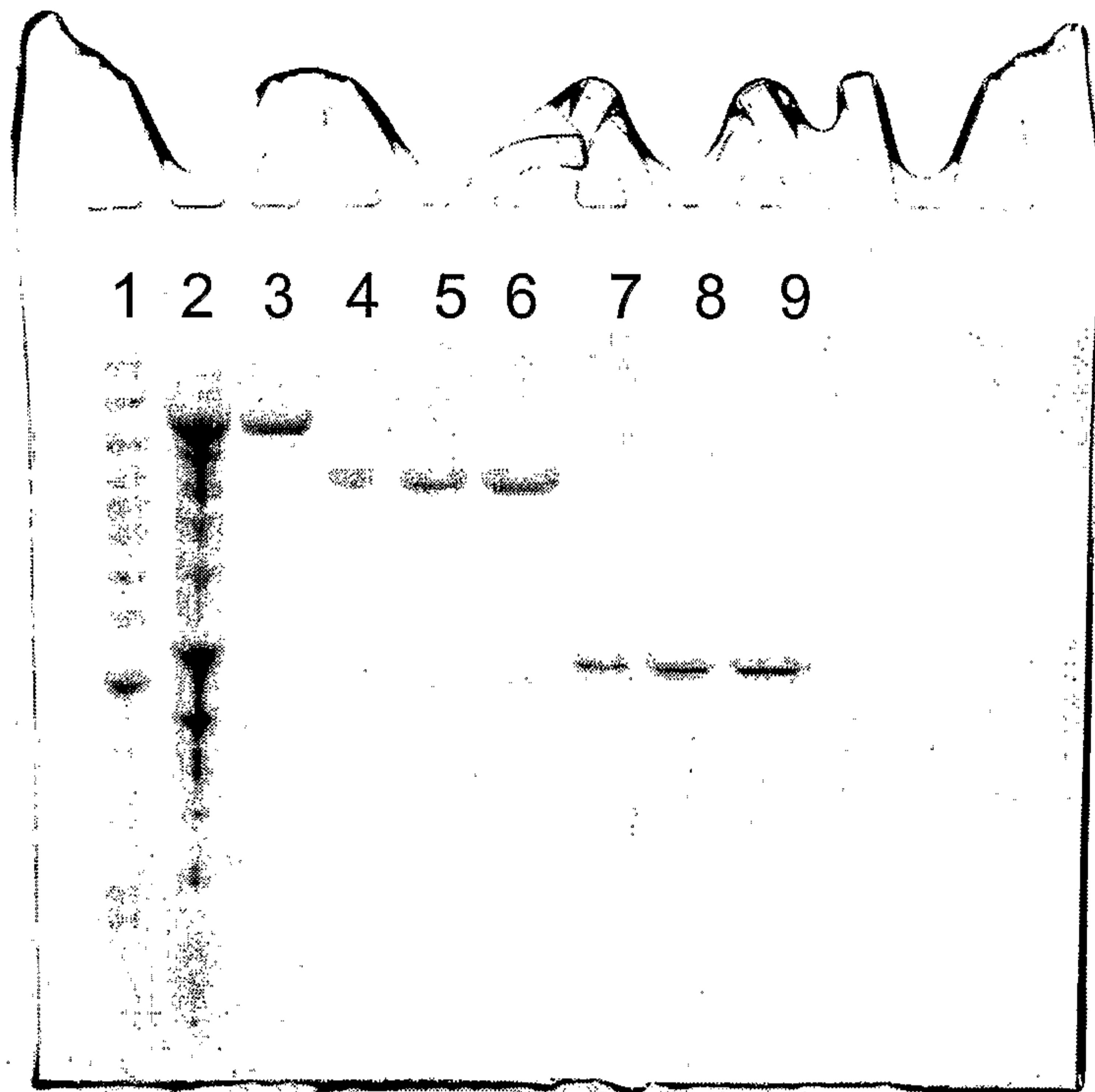
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Figure 17



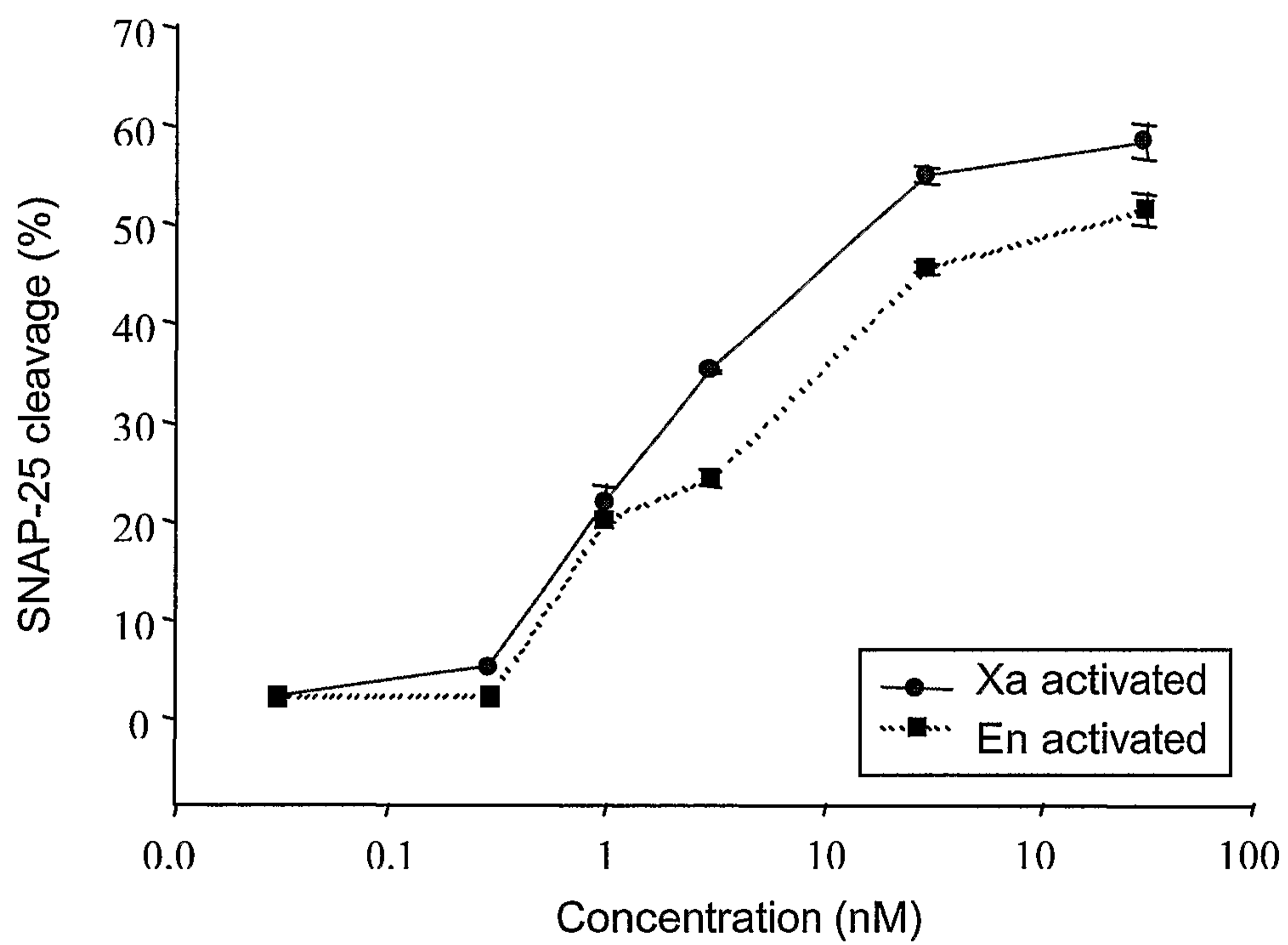
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Figure 18



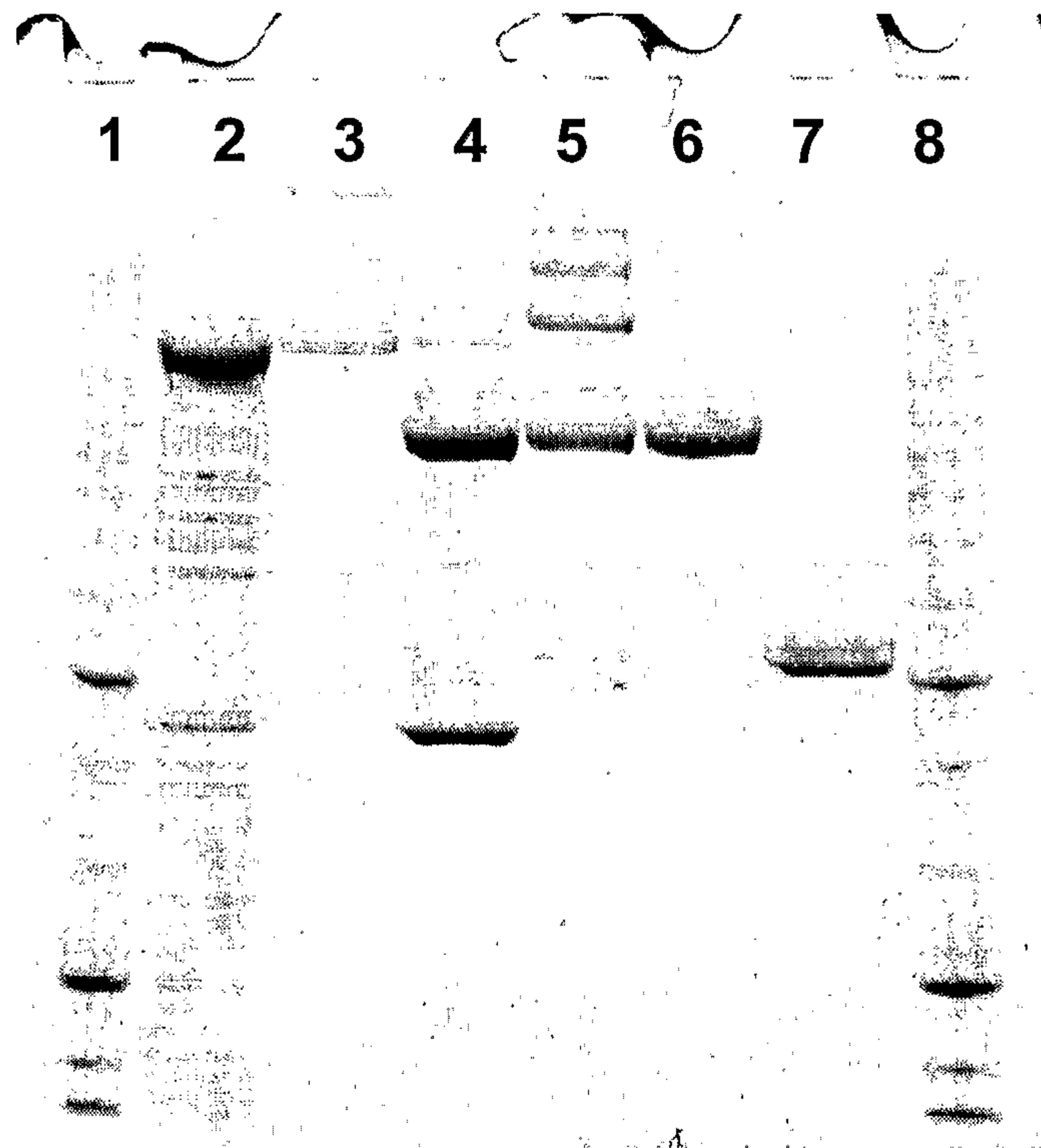
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Figure 19



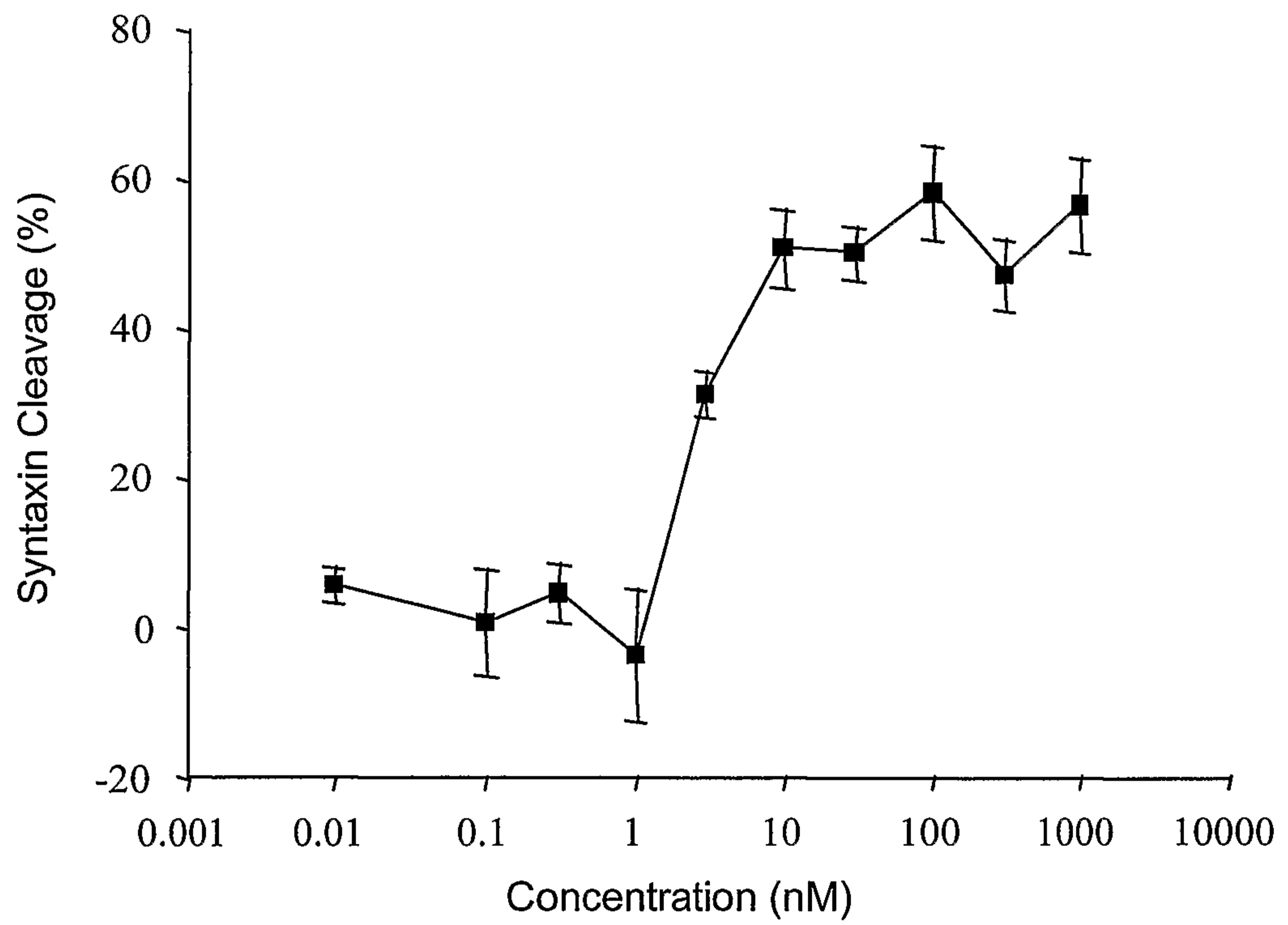
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Figure 20



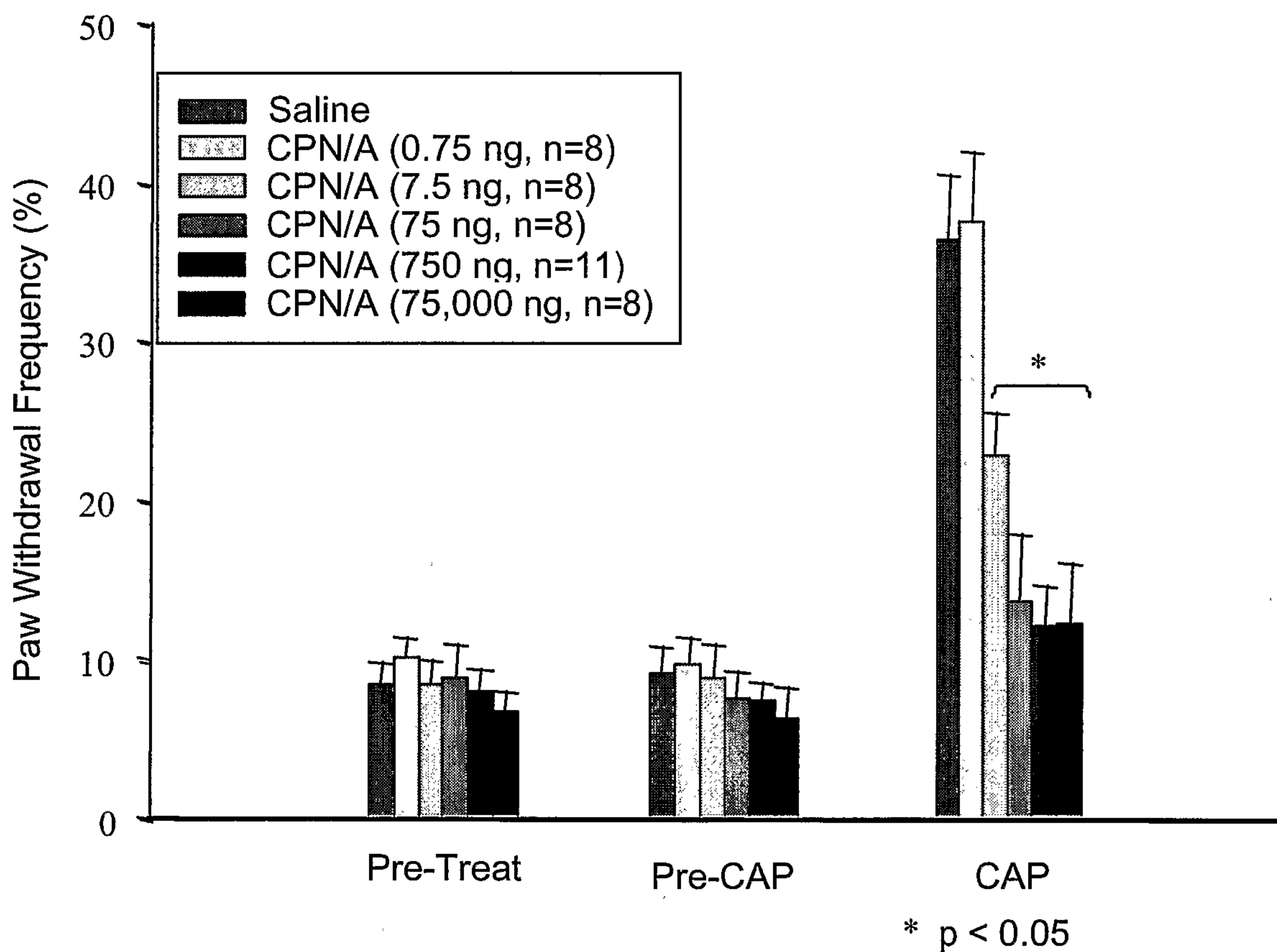
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Figure 21



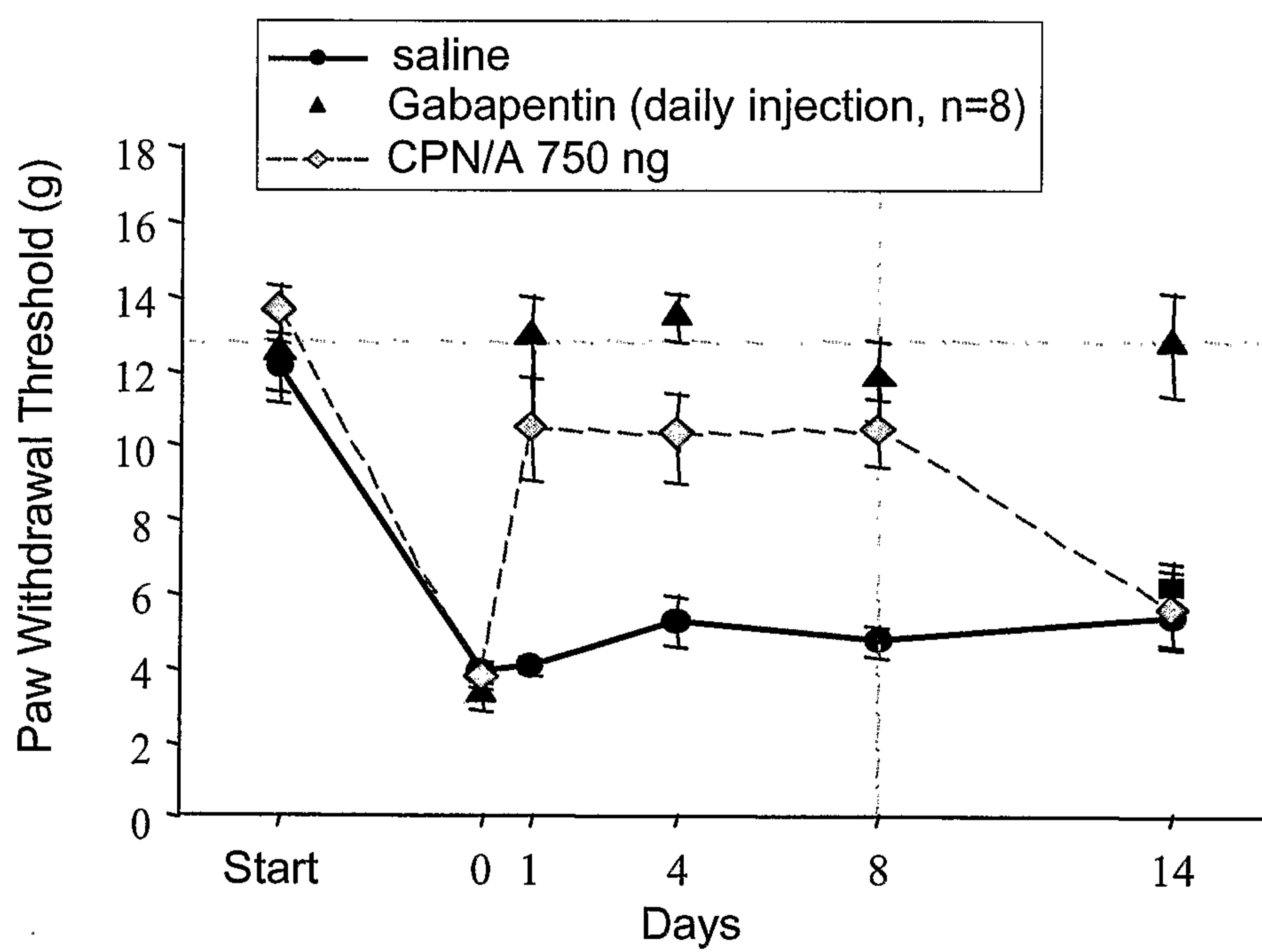
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Figure 22



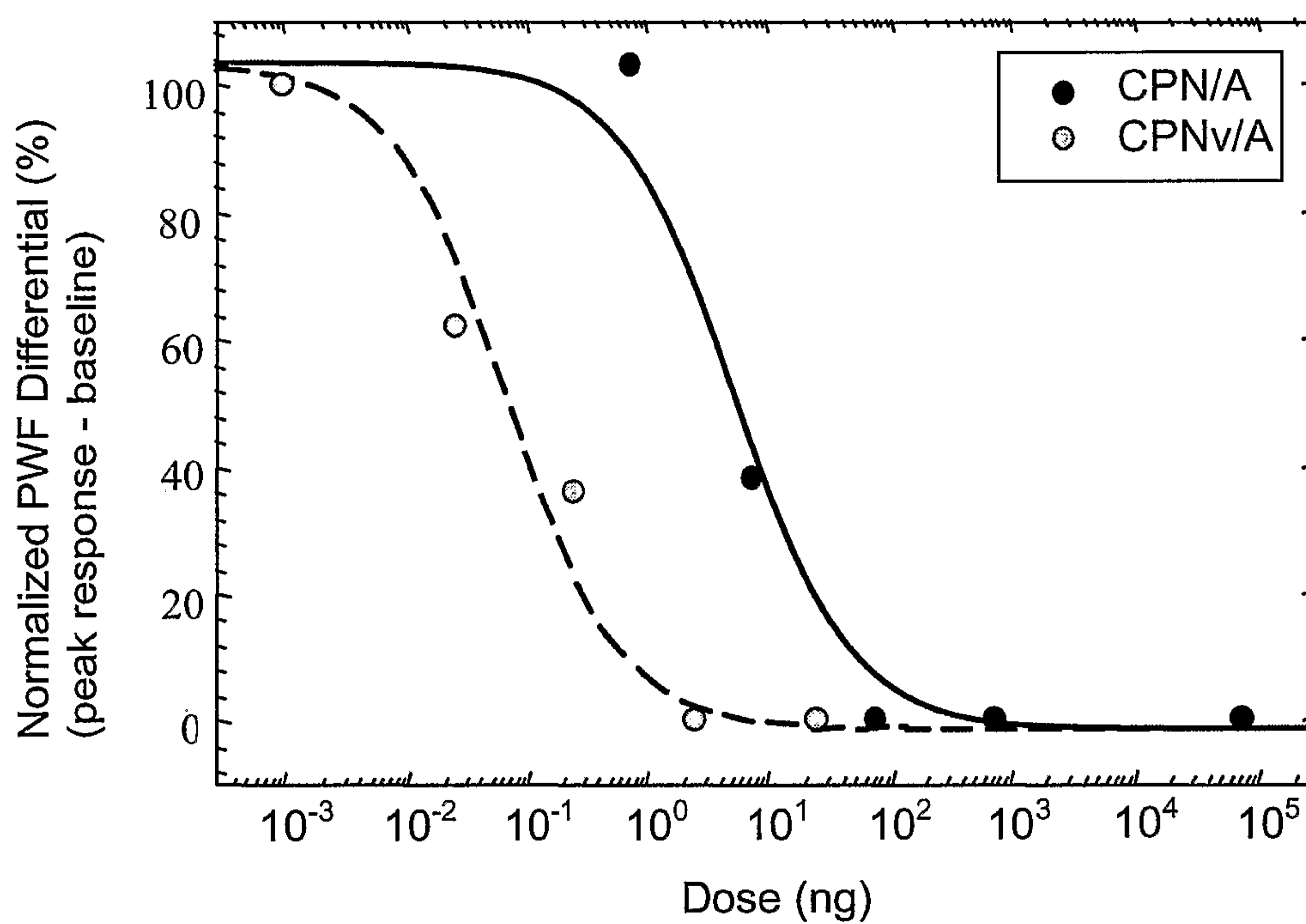
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Figure 23



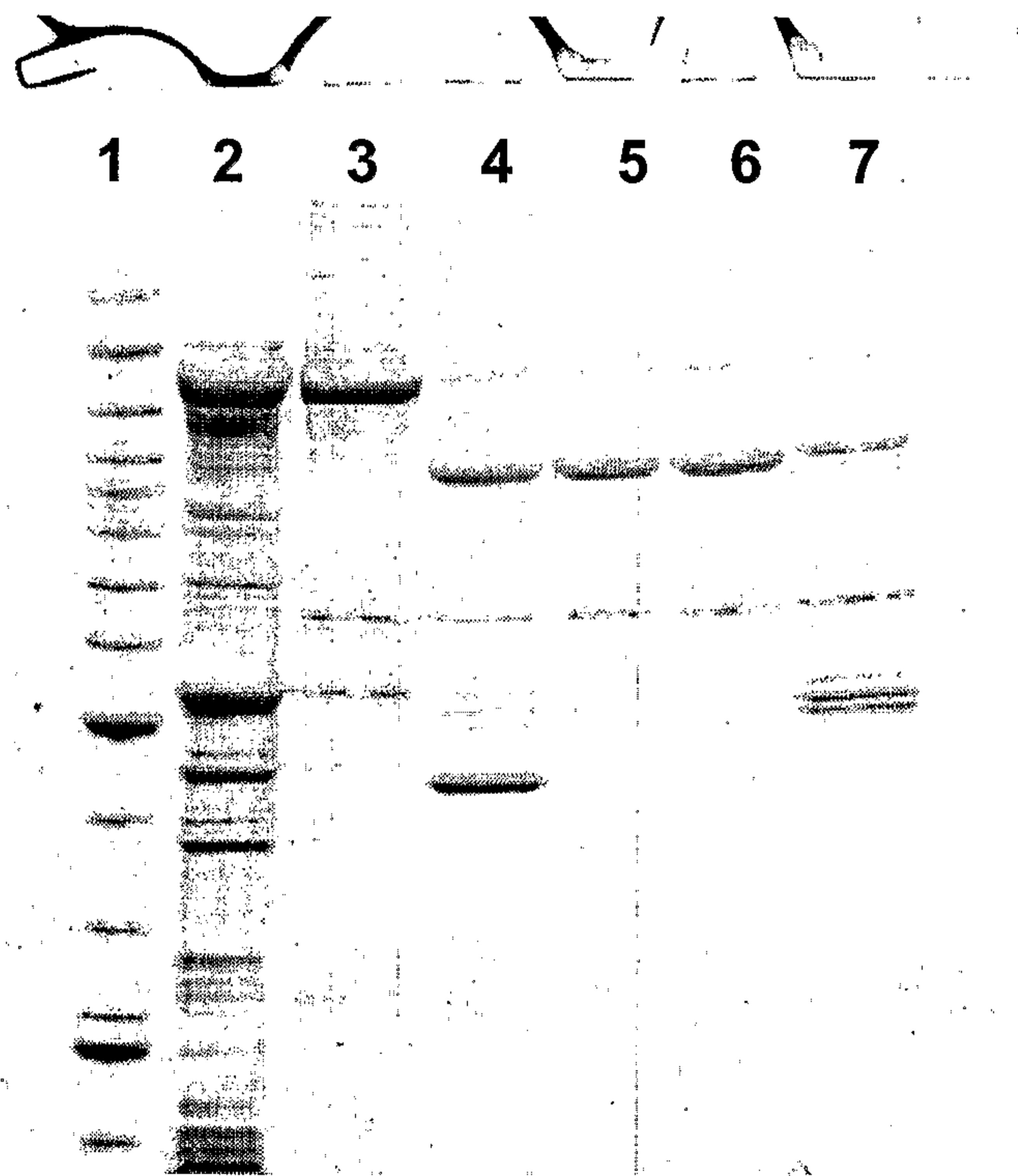
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Figure 24



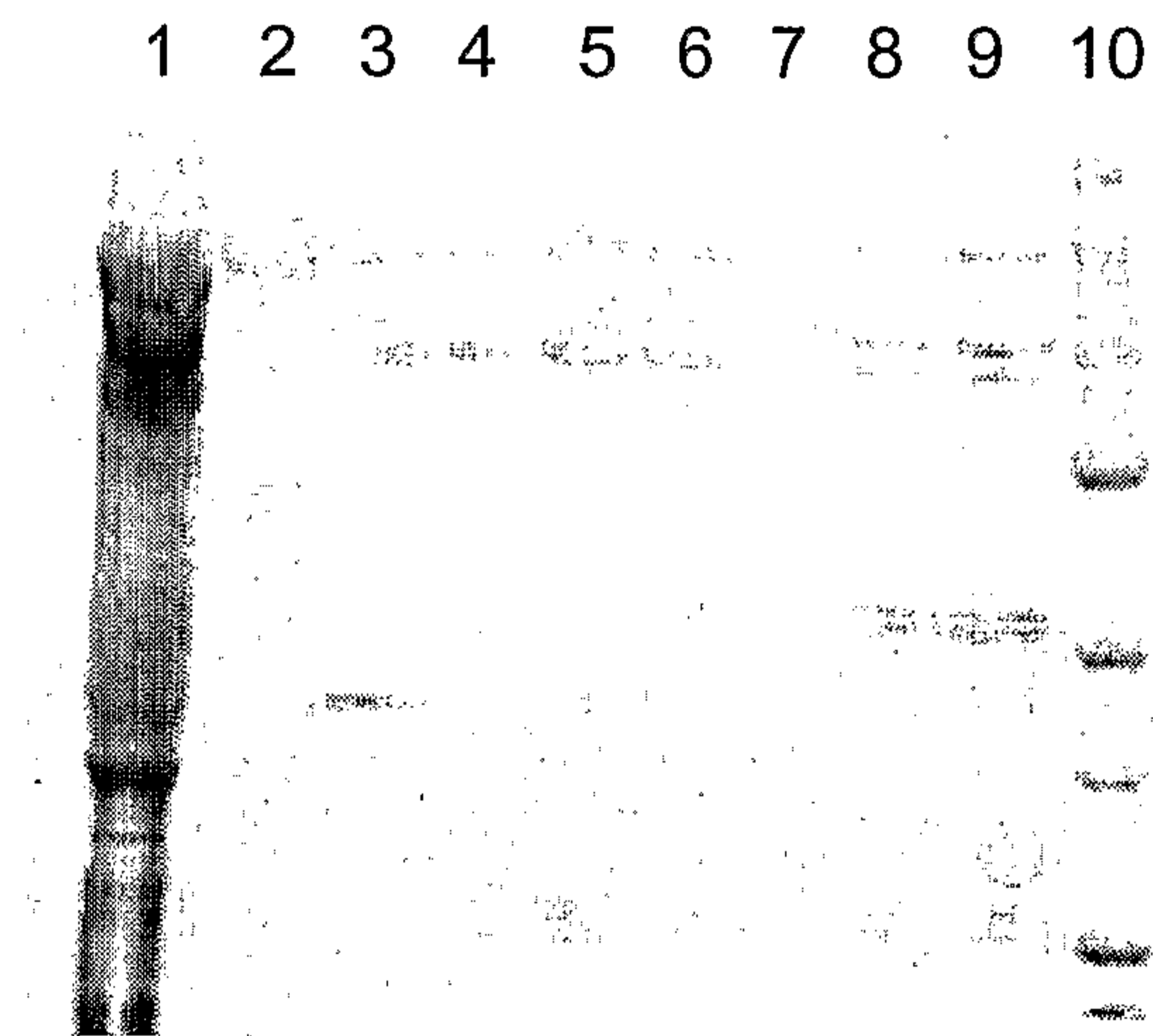
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Figure 25



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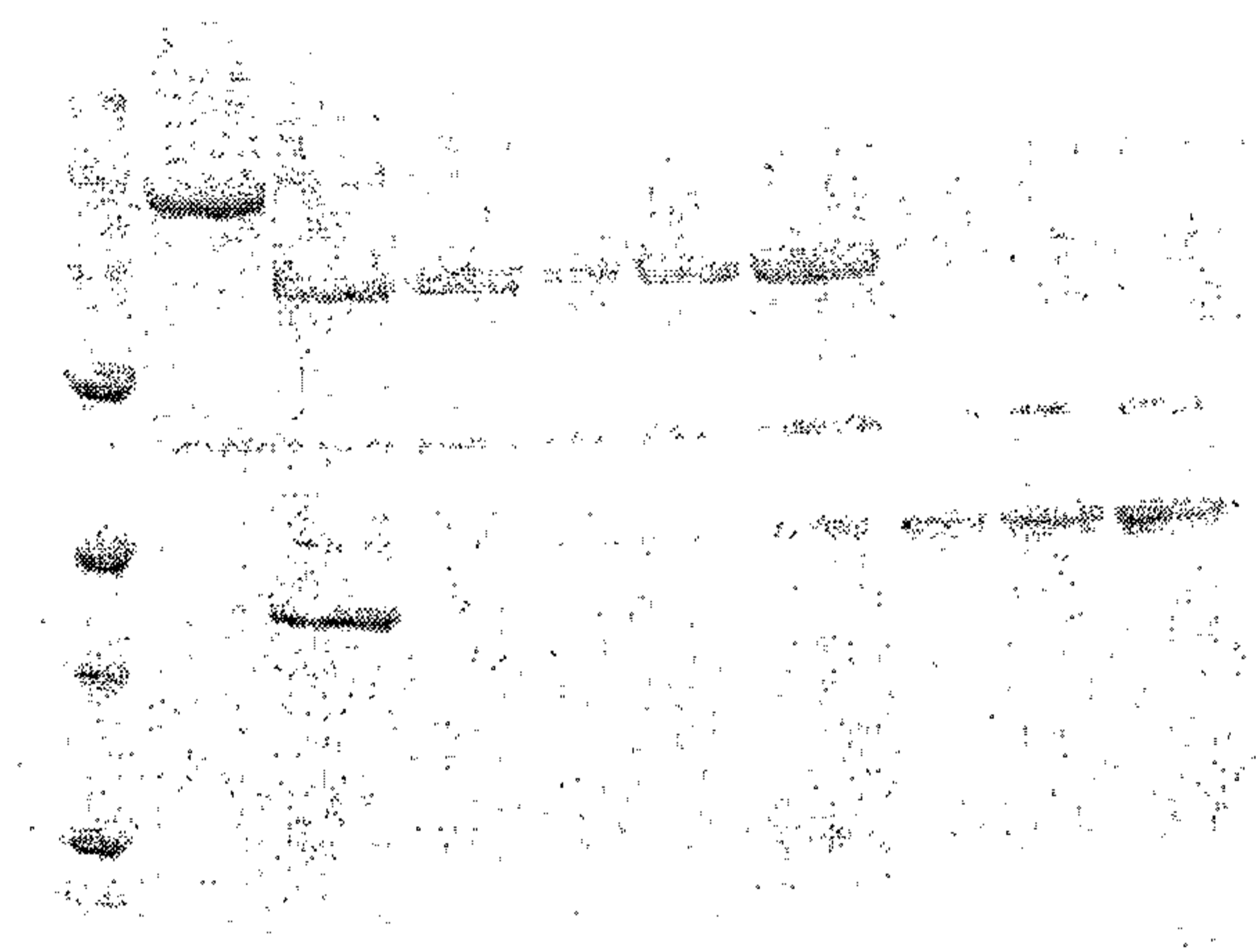
Figure 26



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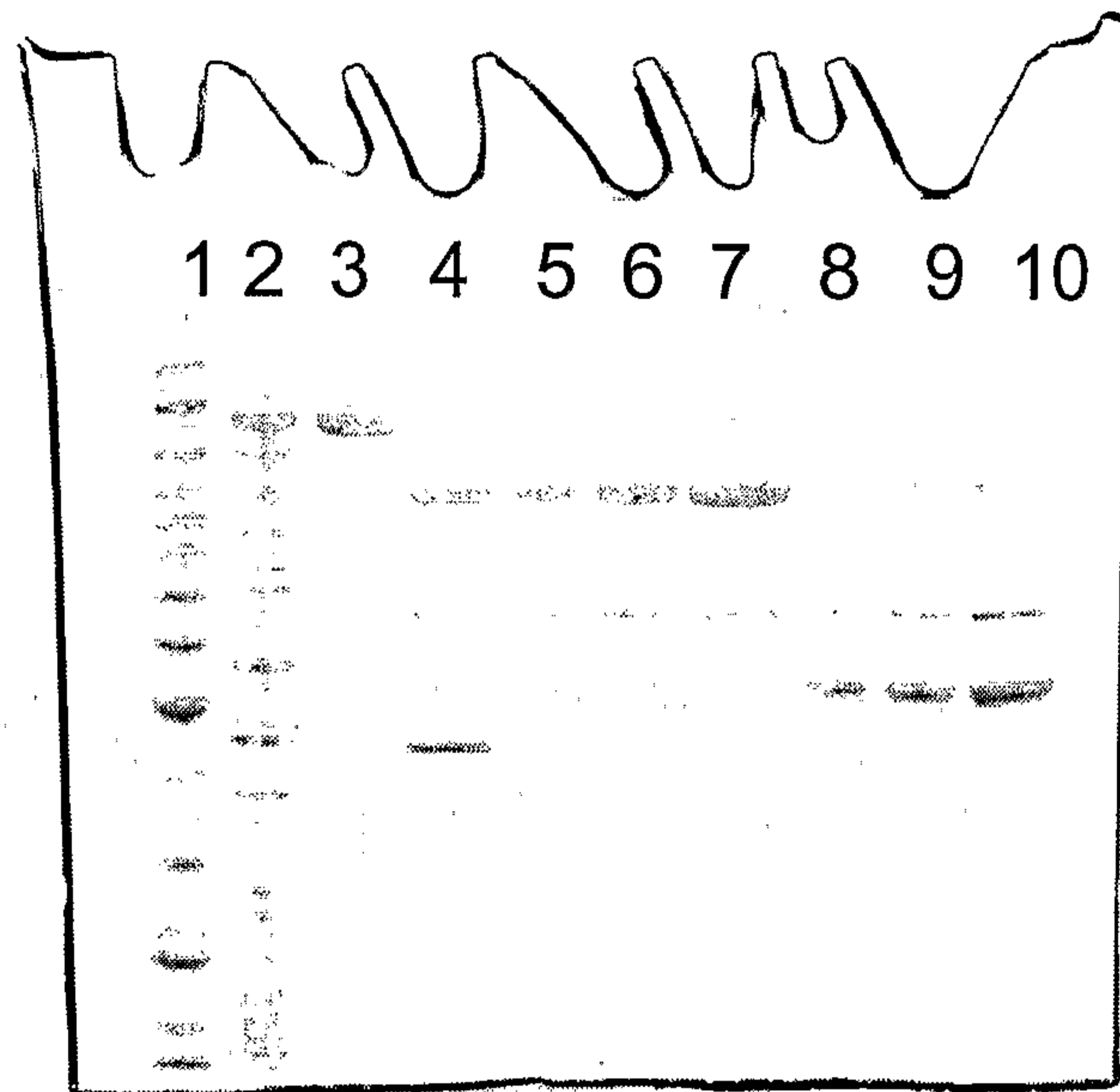
Figure 27

1 2 3 4 5 6 7 8 9 10



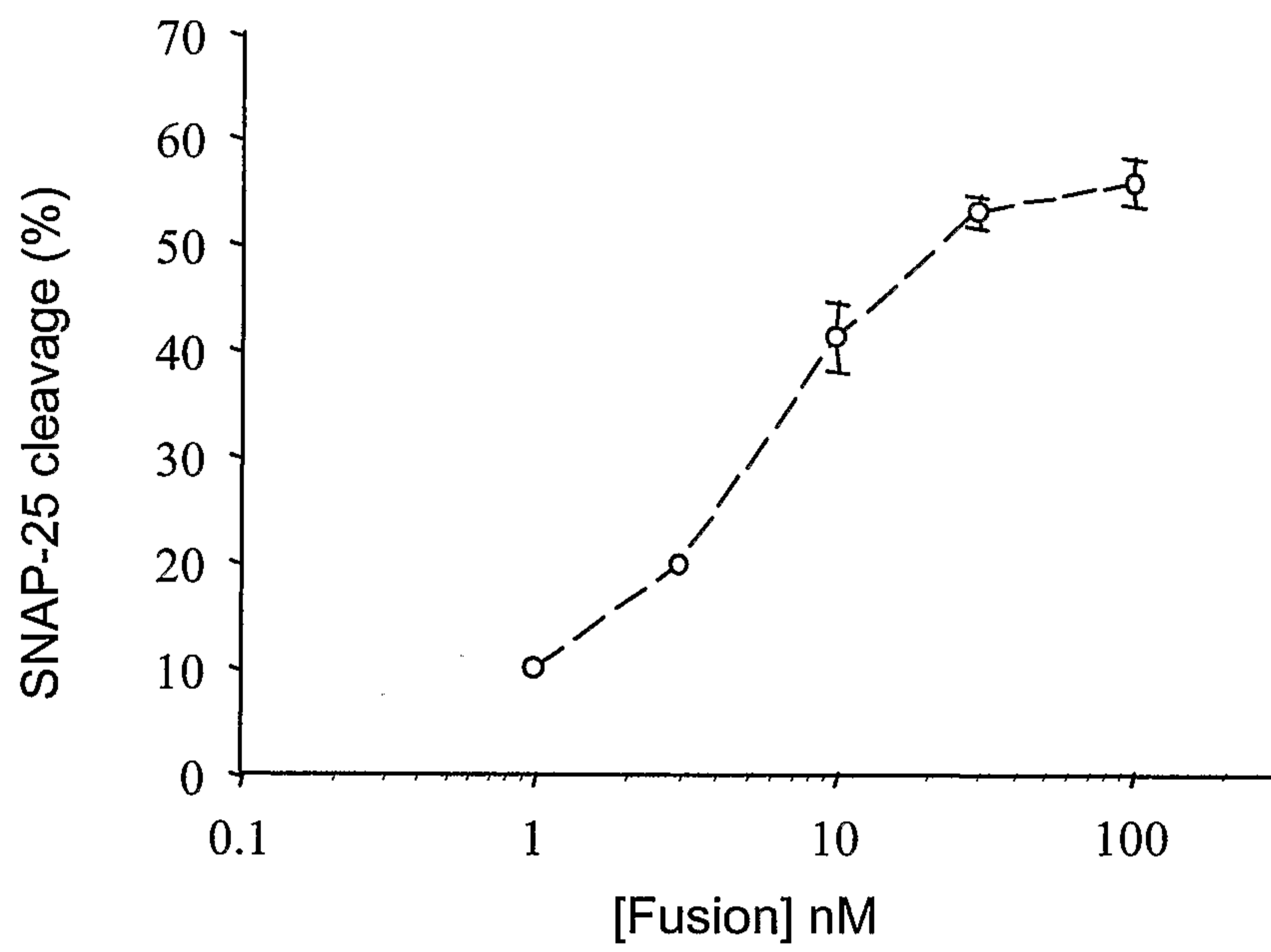
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Figure 28



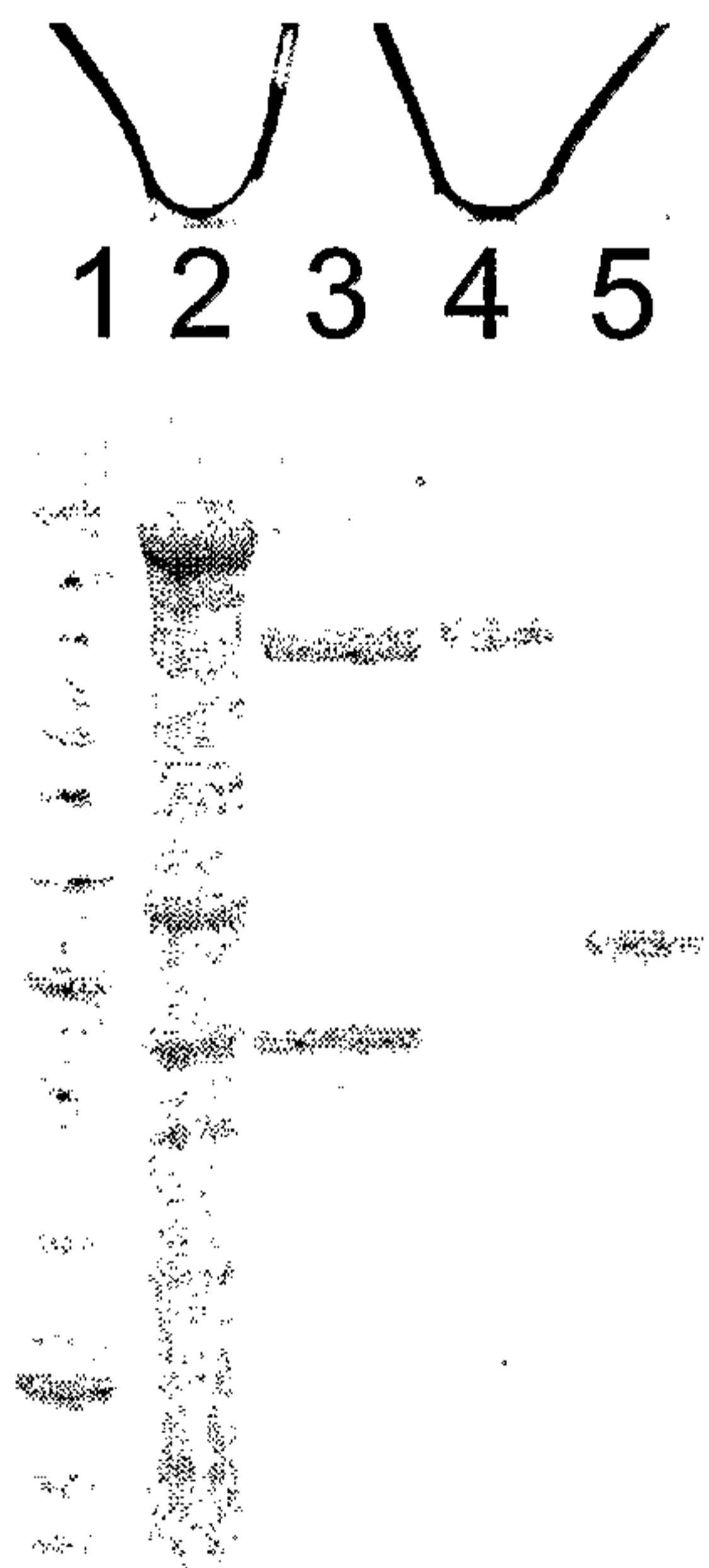
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Figure 29



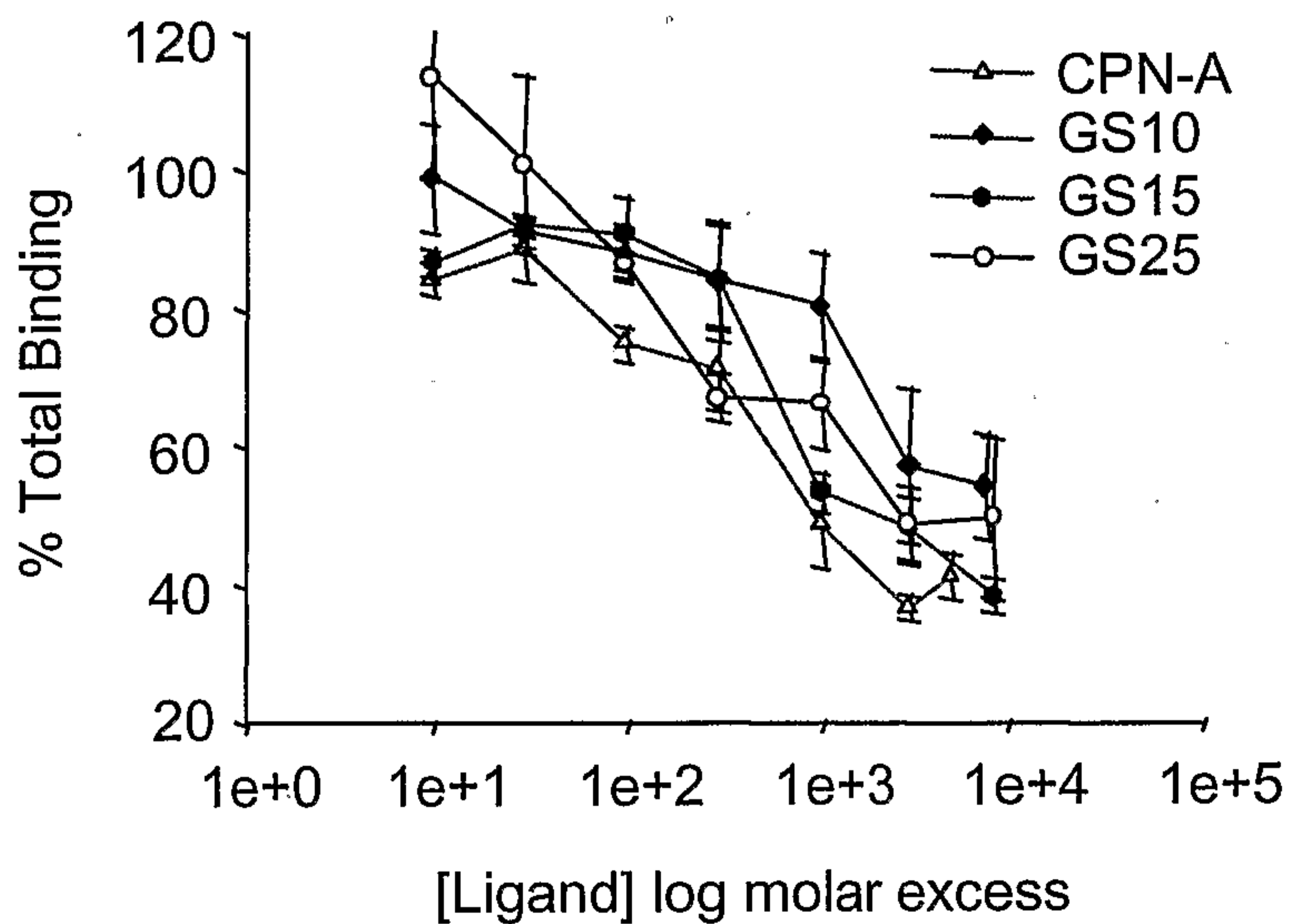
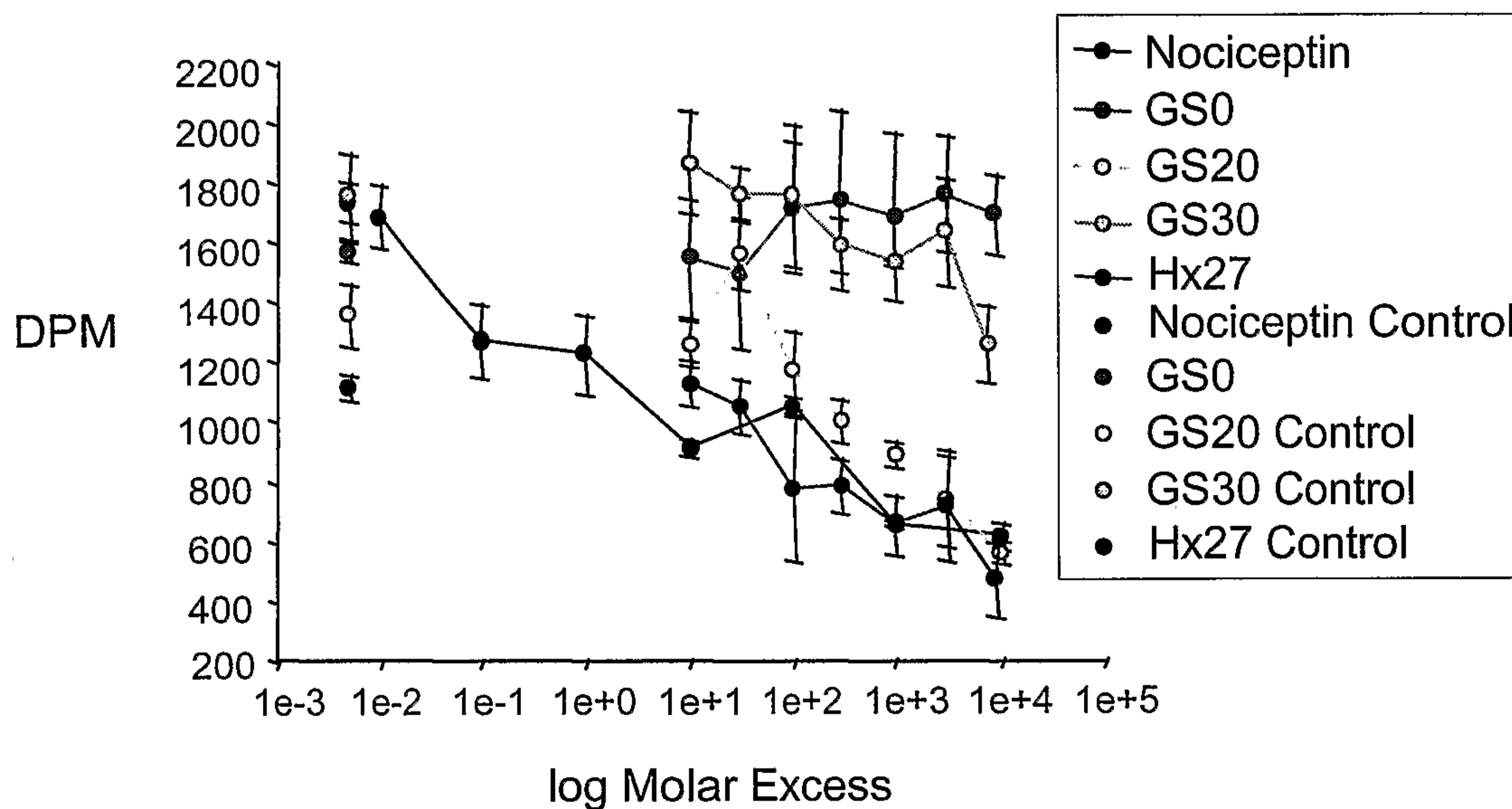
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Figure 30



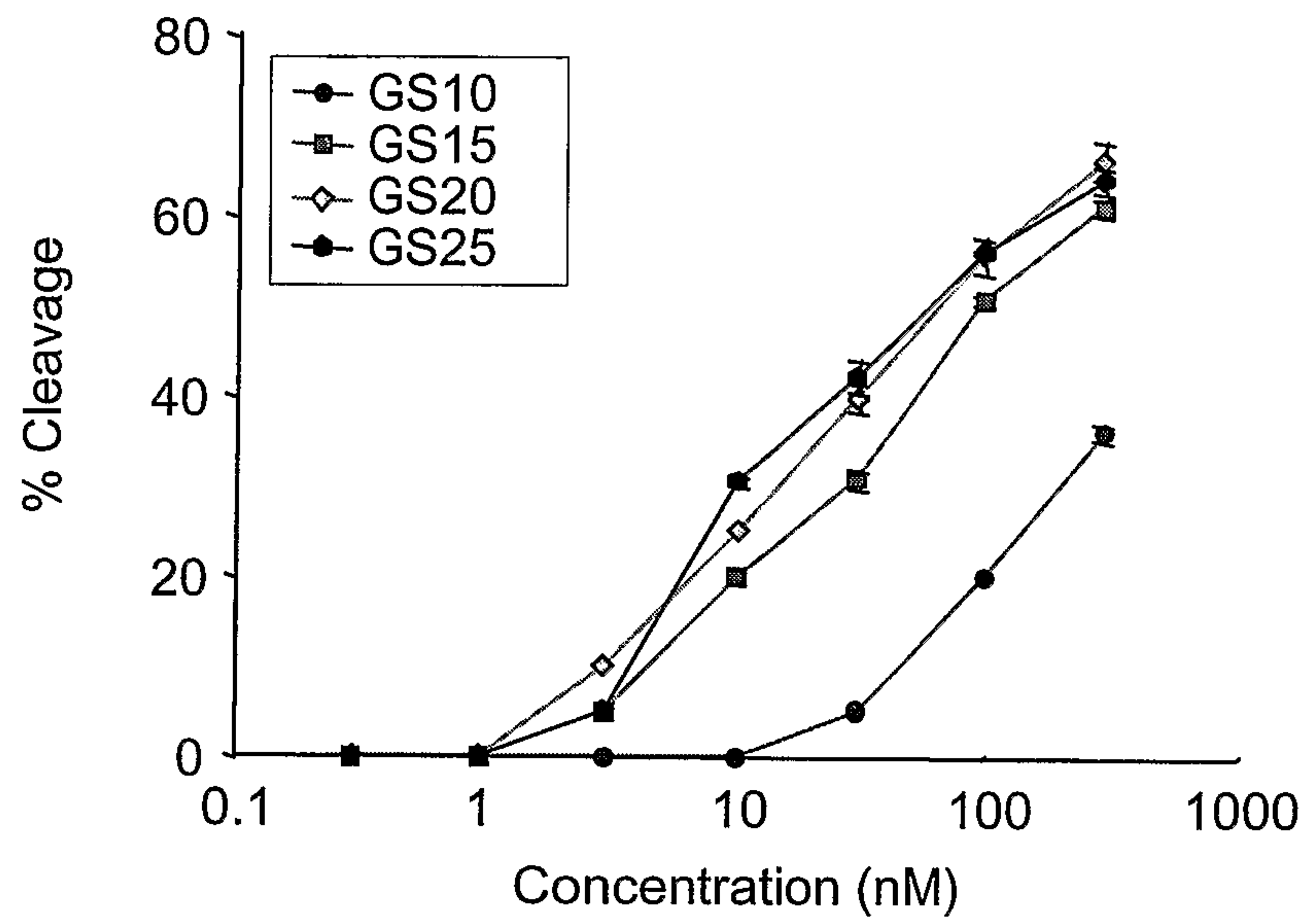
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Figure 31



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Figure 32



Competition Assay : Nociceptin-LH_N/A Fusions
vs 1nM [³H]-Nociceptin on eDRGs (4°C)

