

# JCU ePrints

This file is part of the following reference:

**Shtevi, Abraham (2009) *Design, expression, and testing of a bivalent subunit botulism vaccine for use in cattle*. PhD thesis, James Cook University.**

Access to this file is available from:

<http://eprints.jcu.edu.au/11603>

# References

- Abdul Jabbar M. and Nayak D.P. (1987). Signal processing, glycosylation and secretion of mutant hemagglutinins of a human influenza virus by *Saccharomyces cerevisiae*. *Mol Cell Biol.* 7(4), 1476-1485.
- Aktories K., and Barth H. (2004). The actin-ADP-ribosylating *Clostridium botulinum* C2 toxin. *Anaerobe* 10(2), 101-105.
- Al-Shakhshir R.H., Regnier F.E., White J.L. and Hem S.L. (1995). Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminium-containing adjuvants. *Vaccine* 13(1), 41-44.
- Angov E., Hillier C. J. , Kincaid, R.L. and Lyon J.A. (2008). Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. *PLoS ONE* 3(5), e2189.
- Angulo F.J., Getz J., Taylor J. P., Hendricks K.A., Hatheway C.L., Barth S.S. (1998). A large outbreak of botulism: the hazardous baked potato. *J Infect Dis.* 178(1), 172-177.
- Anonymous (updated 14 January, 2004) Certificate of Analysis – Standard reference material 927c, Bovine Serum Albumin (7% Solution) *Nat. Inst. of Stand. & Tech.* found in site: [http://ts.nist.gov/MeasurementServices/ReferenceMaterials/archived\\_certificates/927c.Jan14,%202004.pdf](http://ts.nist.gov/MeasurementServices/ReferenceMaterials/archived_certificates/927c.Jan14,%202004.pdf)
- Anonymous (01/2005) European Pharmacopoeia 5.0 monograph 0361
- Anonymous (November 25, 2006), Outbreak of botulism in fattening cattle in Scotland. *The Veterinary Record- news item.* 159(22), 729-732.
- Anonymous (updated 2009) Generally Recognized as Safe (GRAS) available online: <http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm>
- Arimitsu H., Lee J.C., Sakaguchi Y., Hayakawa Y., Hayashi M., Nakaura M. (2004). Vaccination with recombinant whole heavy chain fragments of *Clostridium botulinum* Type C and D neurotoxins. *Clin Diagn Lab Immunol.* 11(3), 496-502.
- Bader O. , Krauke Y. , and Hube B. (2008). Processing of predicted substrates of fungal Kex2 proteinases from *Candida albicans*, *C. glabrata*, *Saccharomyces cerevisiae* and *Pichia pastoris*. *BMC Microbiol.* 8, 116.
- Baldwin M.R., Tepp W.H. , Pier C.L. , Bradshaw, M. , Ho M., Wilson B.A. , Fritz R.B. , Johnson E. A. and Barbieri J.T. (2005). Characterization of the antibody response to the receptor binding domain of botulinum neurotoxin serotypes A and E. *Infect Immun.* 73(10), 6998-7005.

- Baldwin M.R., Tepp W.H. , Przedpelski A. , Pier C.L., Bradshaw M., Johnson E.A. (2008). Subunit vaccine against the seven serotypes of botulism. *Infect Immun.* 76(3), 1314-1318.
- Barbier G., Joshi R.C., Campbell E.R. and Campbella W.H. (2004). Purification and biochemical characterization of simplified eukaryotic nitrate reductase expressed in *Pichia pastoris*. *Protein Expression and Purification* 37, 61–71.
- Bardwell J.C. (1994). Building bridges: disulphide bond formation in the cell. *Mol Microbiol.* 14(2), 199-205.
- Barry J, Ward M, Cotter S, MacDiarmada J, Hannan M, Sweeney B, Grant K.A., McKeown P. (2008). Botulism in injecting drug users, Dublin, Ireland. *European Communicable Disease Bulletin* November- December. 14(1) electronic issue.
- Barth H., Aktories K., Popoff M.R., Stiles B.G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. *Microbiol Mol Biol Rev.* 68(3), 373-402.
- Beveridge W.I.B. (1983). Animal Health in Australia volume 4 - Bacterial diseases of Cattle, Sheep, and Goats. Australian Bureau of Animal Health
- Bienvenu J.G. , Morin M. and Forget S. (1990). Quebec. Poultry litter associated botulism (type C) in cattle. *Can Vet J.* 31(10), 711.
- Bigalke H. and Rummel A. (2005). Medical aspects of toxin weapons. *Toxicology.* 214(3), 210-220.
- Böhnel H. , Neufeld B. and Gessler F. (2005). Botulinum neurotoxin type B in milk from a cow affected by visceral botulism. *Vet J.* 169(1), 124-125.
- Böhnel H., Schwagerick B., Gessler F. (2001). Visceral botulism--a new form of bovine Clostridium botulinum toxication. *J Vet Med.* 48(6), 373-83.
- Böhnel H., Wagner C., Gessler F. (2008). Tonsils--place of botulinum toxin production: results of routine laboratory diagnosis in farm animals. *Vet Microbiol.* 130(3-4), 403-9.
- Boroff D. A. and Fleck U. (1966). Statistical analysis of a rapid *in vivo* method for the titration of the toxin of *Clostridium botulinum*. *J Bacteriol.* 92(5), 1580-1581.
- Brankamp R.G., Sreekrishna K., Smith P.L., Blankenship D.T, and Cardin A.D. (1995). Expression of a Synthetic Gene Encoding the Anticoagulant-Antimetastatic Protein Ghilanten by the Methylotropic Yeast *Pichia pastoris*. *Protein Expression and Purification* 6, 813–820.
- Braun U. , Feige K., Schweizer G. and Pospischil A. (2005). Clinical findings and treatment of 30 cattle with botulism. *Vet Rec.* 156(14), 438-441.

- Breidenbach M.A. and Brunger A.T. (2004). Substrate recognition strategy for botulinum neurotoxin serotype A. *Nature* 432(7019), 925-929.
- Bretthauer R.K. (2003). Genetic engineering of *Pichia pastoris* to humanize N-glycosylation of proteins. *Trends in Biotechnology* 21(11), 459-462.
- Brewer J.M. (2006). (How) do aluminium adjuvants work? *Immunol Lett.* 102(1), 10-15.
- Brickman T. J. and Armstrong S. K. (2002). *Bordetella* Interspecies Allelic Variation in AlcR Inducer Requirements: Identification of a Critical Determinant of AlcR Inducer Responsiveness and Construction of an *alcR*(Con) Mutant Allele. *Journal of Bacteriology* 184(6), 1530-1539.
- Bulmer M. (1987) Coevolution of codon usage and transfer RNA abundance. *Nature* 325, 728-730.
- Burnette N.W. (1981). "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry* 112(2), 195-203.
- Byard R.W., Moore L., Bourne A.J., Lawrence A.J., Goldwater P.N. (1992). *Clostridium botulinum* and sudden infant death syndrome: A 10 year prospective study. *J. Paediatr. Child Health* 28, 156-157.
- Byrne M. P. and Smith L. A. (2000). Development of vaccines for prevention of botulism. *Biochimie* 82, 955-966.
- Byrne M.P. , Smith T.J. , Montgomery V.A. and Smith L.A. (1998). Purification , potency and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate. *Infect Immun.* 66(10), 4817-4822.
- Byrne M.P. , Titball R.W. , Holley J. and Smith L.A. (2000). Fermentation, purification, and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from *Pichia pastoris*. *Protein Expression and Purification* 18(3), 327-337.
- Campbell J.B. and Peerbaye Y.A. (1992). Saponin. *Research in Immunology* 143(5), 526-530.
- Cereghino J.L. and Cregg J.M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev.* 24(1), 45-66.
- Claassen E. , Boersma W.J.A. (1992). Characteristics and practical use of new-generation adjuvants as an acceptable alternative to Freund's complete adjuvant. *Res. Immunol.* 143, 475-502.

Clare J.J., Rayment F.B. , Ballantine S.P. , Sreekrishna K. and Romanos M.A. (1991). High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology (N Y)* 9(5), 455-460.

Clayton M.A. , Clayton J.M. , Brown D.R. and Middlebrook J.L. (1995). Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*. *Infect Immun.* 63(7), 2738-2742.

Coffield J.A. , Bakry N. , Zhang R.D. , Carlson J. , Gomella L.G. and Simpson L.L. (1997). *In Vitro* Characterization of Botulinum Toxin Types A, C and D. Action on Human Tissues: Combined Electrophysiologic, Pharmacologic and Molecular Biologic Approaches. *The Journal Of Pharmacology And Experimental Therapeutics* 280, 1489–1498.

Couderc R. and Baratti J. (1980). Oxidation of methanol by the yeast *Pichia pastoris*. Purification and properties of alcohol oxidase. *Agric. Biol. Chem.* 44, 2279-2289.

Cregg J.M. , Vedvick T.S. and Raschke W.C. (1993). Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology (N Y)* 11(8), 905-910.

Critchley E. M. R. (1991). A comparison of human and animal botulism: a review. *Journal of the Royal Society of Medicine* 84, 295-298.

Daly R. and Hearn M.T. (2006) . Expression of the human activin type I and II receptor extracellular domains in *Pichia pastoris*. *Protein Expression and Purification.* 46(2), 456–467.

Daly R. and Hearn M.T. (2005). Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *J Mol Recognit.* 18(2), 119-138.

Damasceno L.M, Pla I. , Chang H.J. , Cohen L. , Ritter G. , Old L. J. and Battc C.A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. *Protein Expression and Purification* 37, 18–26.

DasGupta B.R. and Boroff D.A. (1968). Separation of toxin and hemagglutinin from crystalline toxin of *Clostridium botulinum* type A by anion exchange chromatography and determination of their dimensions by gel filtration. *J Biol Chem.* 243(5), 1065-72.

Demarchi J., Mourgues C., Orio J, Prevot A.R. (1958). Existence du botulisme humain de type D. *Bulletin de l'Academie Nationale de Medecine* 142, 580-582.

DePaz R.A. , Henderson I. and Advant S. J. (2005). Formulation of botulinum neurotoxin heavy chain fragments for vaccine development: mechanisms of adsorption to an aluminum-containing adjuvant. *Vaccine* 23(31), 4029-4035.

- Dingermann T. (2008). Recombinant therapeutic proteins: Production platforms and challenges. *Biotechnol. J.* 3, 90–97.
- Dobereiner J. , Tokarnia C.H. , Langenegger J. and Dutra I.S. (1992). Epizootic botulism of cattle in Brazil. *Dtsch Tierarztl Wochenschr.* 99(5), 188-190.
- Domingo R.M. MD, Haller J.S. MD, and Gruenthal M. MD, PhD (2008). Infant Botulism: Two Recent Cases and Literature Review. *Journal of Child Neurology* 23(11), 1336-1346.
- Dong M., Yeh f., Tepp W.H., Dean C., Johnson E.A, Janz R., Chapman E.R. (2006). SV2 Is the Protein Receptor for Botulinum Neurotoxin A. *Science* 312, 592-596.
- Dong M., Richards D.A., Goodnough M.C., Tepp W.H., Johnson E.A. and Chapman E.R. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J Cell Biol.* 162(7), 1293-1303.
- Doutre M.P. (1969). Frequency of animal botulism from hydric origin in Senegal (article in French). *Rev Elev Med Vet Pays Trop.* 22(1), 29-31.
- Dus Santos M.J. and Wigdorovitz A. (2005). Transgenic plants for the production of veterinary vaccines. *Immunol Cell Biol.* 83(3), 229-238.
- Dux M.P., Barent R., Sinha J., Gouthro M., Swanson T., Barthuli A. (2006). Purification and scale-up of a recombinant heavy chain fragment C of botulinum neurotoxin serotype E in *Pichia pastoris* GS115. *Protein Expression and Purification* 45(2), 359-367.
- Egyed M.N., Shlosberg A., Klopfer U. (1978). Mass outbreaks of Botulism in ruminants associated with ingestion of feed containing poultry waste. *Refuah vet.* 35, 93-104.
- Eklund M.W. and Poysky F.T. (1974). Interconversion of type C and D strains of *Clostridium botulinum* by specific bacteriophages *Applied Microbiology* 27(1), 251-258.
- Engvall E., Perlmann P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* 8(9), 871-874.
- Esposito V.M., Feeley J.C., Leeder W.D., Pittman M. (1969). Immunological Response of Three Mouse Strains to Typhoid Vaccine and Vi Antigen. *J Bacteriol.* 99(1), 8–12.
- Fairweather N.F., Lyness V.A., Pickard D.J., Allen G., and Thomson R.O. (1986). Cloning, nucleotide sequencing and expression of tetanus toxin fragment c in *Escherichia coli*. *J Bacteriol.* 165(1), 21-27.
- Fairweather N. F., Lyness V.A., and Maskell D. J. (1987). Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli*. *Infect Immun.* 55(11), 2541-5.
- Fenicia L., Anniballi F., Aureli P., Clin E.J (2007). Intestinal toxemia botulism in Italy, 1984–2005. *Microbiol Infect Dis.* 26, 385–394.

Fujinaga Y. (2006). Transport of bacterial toxins into target cells: pathways followed by cholera toxin and botulinum progenitor toxin. *J Biochem.* 140(2), 155-160.

Galey F.D., Terra R., Walker R., Adaska J., Etchebarne M.A., Puschner B. (2000). Type C botulism in dairy cattle from feed contaminated with a dead cat. *J Vet Diagn Invest.* 12(3), 204-209.

Gellissen G., and Hollenberg C. P. (1997). Application of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis* - a review. *Gene* 190, 87-97.

Gellissen G., Kunze G., Gaillardin C., Cregg J. M., Berardi E., Veenhuis M., van der Klei I. (2005). New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adenivorans* and *Yarrowia lipolytica* – A comparison. *FEMS Yeast Research* 5, 1079–1096.

Gibson, L.A. (1986). Botulism in dairy cows. *Vet Rec.* 118 (11), 309.

Glick B.S. and Rothman J.E. (1987). Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature* 326(6110), 309-312.

Graham R. and Schwarze H.R. (1921). Botulism in Cattle. *J Bacteriol.* 6(1), 69-83.

Guarna M.M., Lesnicki G.J., Tam B.M., Robinson J., Radziminski C. Z., Hasenwinkle D. (1997). On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. *Biotechnol Bioeng.* 56(3), 279-286.

Gustafsson C., Govindarajan S., and Minshull J. (2004). Codon bias and heterologous protein expression. *Trends Biotechnol.* 22(7), 346-353.

Gustafson G.L. and Rhodes M.J. (1992). Bacterial cell wall products as adjuvants: early interferon gamma as a marker for adjuvants that enhance protective immunity. *Res. Immunol.* 143, 483–488.

Hanes J., Chiba M., Langer R. (1995). Polymer microspheres for vaccine delivery. *Pharm Biotechnol.* 6, 389-412.

Hansson M., Nygren P.A. and Stahl S. (2000). Design and production of recombinant subunit vaccines. *Biotechnol Appl Biochem.* 32(Pt 2), 95-107.

Hatfield D.L., Lee B.J., Pirtle R.M. (1992). Transfer RNA in protein synthesis CRC Press US. Ikemura T. Ch. 3 Correlation between codon usage and tRNA content in microorganisms 87-120.

Hatfield G.W. and Roth D.A. (2007). Optimizing scaleup yield for protein production: Computationally Optimized DNA Assembly (CODA) and Translation Engineering. *Biotechnol Annu Rev.* 13, 27-42.

- Hatheway C. L. (1990). Toxigenic clostridia. *Clin Microbiol Rev.* 3(1), 66-98.
- Haworth EA., Booy R., Stirzaker L., Wilkes S. and Battersby A. (1993) Is the cold chain for vaccines maintained in general practice? *BMJ.* 307(6898), 242-244.
- Heider L.C., McClure J.T., and Leger E.R. (2001). Presumptive diagnosis of *Clostridium botulinum* type D intoxication in a herd of feedlot cattle. *Can Vet J.* 42(3), 210-212.
- Henikoff S. and Henikoff J. G. (1992). Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA* 89, 10915-10919.
- Higgins D.R. and Cregg J. M. (1998) *Pichia* Protocols, Published by Humana Press, (1998)
- Hong W. (2005). SNAREs and traffic. *Biochim Biophys Acta.* 1744(3), 493-517.
- Horwitz M.A., Marr J. S., Merson M.H., Dowell V.R. and Ellis J.M. (1975). A continuing common-source outbreak of botulism in a family. *Lancet* 2(7940), 861-863.
- Humphreys D.P., Sehdev M., Chapman A.P., Ganesh R., Smith B.J., King L.M., Glover D.J., Reeks D.G., and Stephens P.E. (2000). High-Level Periplasmic Expression in *Escherichia coli* Using a Eukaryotic Signal Peptide: Importance of Codon Usage at the 5' end of the Coding Sequence. *Protein Expression and Purification* 20, 252-264.
- Huss H.H (1980). Distribution of *Clostridium botulinum*. *Applied and Environmental Microbiology* 39(4), 764-769.
- Imperiali B. and O'Connor SE. (1999). Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr Opin Chem Biol.* 3(6), 643-9.
- Inan M., Aryasomayajula D., Sinha J. and Meagher M.M. (2006). Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. *Biotechnol Bioeng.* 93(4), 771-778.
- Inan M. and Meagher M M. (2001). The effect of ethanol and acetate on protein expression in *Pichia pastoris*. *J Biosci Bioeng.* 92(4), 337-341.
- Inoue K., Fujinaga Y., Honke K., Yokota K., Ikeda T., Ohyama T. (1999). Characterization of haemagglutinin activity of *Clostridium botulinum* type C and D 16S toxins, and one subcomponent of haemagglutinin (HA1). *Microbiology* 145(Pt 9), 2533-2542.
- Irwin B., Heck J.D., and Hatfield G.W. (1995). Codon pair utilization biases influence translational elongation step times. *J Biol Chem.* 270(39), 22801-22806.
- Jean D., Fecteau G., Scott D., Higgins R. and Quessy S. (1995). *Clostridium botulinum* type C intoxication in feedlot steers being fed ensiled poultry litter. *Can Vet J.* 36(10), 626-628.



- Jin R., Sikorra S., Stegmann C.M., Pich A., Binz T., and Brunger A.T. (2007). Structural and biochemical studies of botulinum neurotoxin serotype C1 light chain protease: implications for dual substrate specificity. *Biochemistry* 46(37), 10685-10693.
- Johnson S.K., Zhang W., Smith L.A., Hywood-Potter K.J., Todd Swanson S., Schlegel V.L., Meagher M.M. (2003). Scale-up of the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in *Pichia pastoris*. *Protein Expression and Purification* 32, 1–9.
- Kalb S.R., Moura H., Boyer A.E., McWilliams L.G., Pirkle J.L., and Barr J.R. (2006). The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. *Anal Biochem.* 351(1), 84-92.
- Kalka-Moll W.M., Aurbach U., Schaumann R., Schwarz R., Seifert H. (2007). Wound botulism in injection drug users. *Emerg Infect Dis.* 13(6), 942-943.
- Kane J.F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr Opin Biotechnol.* 6(5), 494-500.
- Kane J.F. and Hartley D.L. (1988). Formation of recombinant protein inclusion bodies in *Escherichia coli*. *Trends Biotechnol.* 6, 95-101.
- Kelch W.J., Kerr L.A., Pringle J.K., Rohrbach B.W., and Whitlock R.H. (2000). Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. *J Vet Diagn Invest.* 12(5), 453-455.
- Kimura K., Fujii N., Tsuzuki K., Murakami T., Indoh T., Yokosawa N. (1991). Cloning of the structural gene for *Clostridium botulinum* type C1 toxin and whole nucleotide sequence of its light chain component. *Appl Environ Microbiol.* 57(4), 1168-1172.
- Kimura K., Fujii N., Tsuzuki K., Murakami T., Indoh T., Yokosawa N. (1990). The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. *Biochem Biophys Res Commun.* 171(3), 1304-1311.
- Klebe R.J., Harriss J.V., Sharp Z.D., and Douglas M.G. (1983). A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* 25(2-3), 333-341.
- Kobayashi R., Kohda T., Kataoka K., Ihara H., Kozaki S., Pascual D. W., Staats H. F., Kiyono H., McGhee J. R., and Fujihashi K. (2005). A Novel Neurotoxoid Vaccine Prevents Mucosal Botulism. *The Journal of Immunology* 174, 2190–2195.
- Komar A.A., Lesnik T., and Reiss C. (1999). Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation. *FEBS Letters* 462, 387-391.
- Lacy D. B. and Stevens R.C. (1999). Sequence homology and structural analysis of the clostridial neurotoxins. *J Mol Biol.* 291(5), 1091-1104.

- Lalli G., Bohnert S., Deinhardt K., Verastegui C., and Schiavo G. (2003). The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol.* 11(9), 431-437.
- Lalli G., Herreros J., Osborne S.L., Montecucco C., Rossetto O., and Schiavo G. (1999). Functional characterisation of tetanus and botulinum neurotoxins binding domains. *J Cell Sci.* 112(Pt 16), 2715-2724.
- Lee C.Y., Nakano A., Shiomi N., Lee E.K., Katoh S. (2003). Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. *Enz Microb Technol.* 33, 358–365.
- Lee J.C., Hwang H.J., Sakaguchi Y., Yamamoto Y., Arimitsu H., Tsuji T., Watanabe T., Ohyama T., Tsuchiya T., Oguma K. (2007). C terminal half fragment (50 kDa) of heavy chain components of Clostridium botulinum type C and D neurotoxins can be used as an effective vaccine. *Microbiol Immunol.* 51(4), 445-55.
- Linder S., Schliwa M., Kube-Granderrath E. (1996). Direct PCR Screening of *Pichia pastoris* Clones. *BioTechniques* 20(6), 980-982.
- Lueking A., Holz C., Gotthold C., Lehrach H., and Cahill D. (2000). A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. *Protein Expression and Purification* 20(3), 372-378.
- Mack M.A., Wannemacher M., Hobl B., Pietschmann P., Hock B. (2009). Comparison of two expression platforms in respect to protein yield and quality: *Pichia pastoris* versus *Pichia angusta*. *Protein Expression and Purification* 66(2):165-71.
- Madzak C., Gaillardin C., Beckerich J.M. (2004). Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *Journal of Biotechnology* 109, 63–81.
- Makoff A.J., Oxeer M.D., Romanos M.A., Fairweather N.F., Ballantine S. (1989). Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. *Nucleic Acids Res.* 17(24), 10191-202.
- Maksymowych A.B., Reinhard M., Malizio C.J., Goodnough M.C., Johnson E.A., and Simpson L.L. (1999). Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. *Infect Immun.* 67(9), 4708-4712.
- Maloy S. R., Cronan J. E. and Freifelder D. (1994), *Microbial Genetics* 2<sup>nd</sup> Edition: illustrated, Published by Jones & Bartlett Publishers.
- Mansur M., Cabello C., Hernández L., País J., Varas L., Valdés J., Terrero Y., Hidalgo A., Plana L., Besada V., García L., Lamazares E., Castellanos L., Martínez E. (2005). Multiple gene copy number enhances insulin precursor secretion in the yeast *Pichia pastoris*. *Biotechnol Lett.* 27(5), 339-45.

- Marin M. (2008). Folding at the rhythm of the rare codon beat. *Biotechnol J.* 3, 1047–1057.
- Marston F.A. (1986). The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem J.* 240(1), 1-12.
- Martin S. (2003). Clostridium botulinum type D intoxication in a dairy herd in Ontario. *Can Vet J.* 44(6), 493-495.
- Martinet W., Saelens X., Deroo T., Neiryneck S., Contreras,R., Min Jou W. (1997). Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase. *Eur J Biochem.* 247(1), 332-338.
- Meyer K.P. (1956). The status of botulism as a world health problem. *Bull World Health Organ.* 15, 281-98.
- Midura T.F., Snowden S., Wood R.M., Clin J. and Arnon\_S.S. (1979). Isolation of *Clostridium botulinum* from Honey. *Microbiol.* 9(2), 282–283.
- Mitraki A. and King J. (1989). Protein folding intermediates and inclusion body formation. *Biotechnology (NY)* 7, 690-697.
- Miyata K., Yoneyama T., Suzuki T., Kouguchi H., Inui K., Niwa K., Watanabe T., Ohyama T. (2009). Expression and stability of the nontoxic component of the botulinum toxin complex. *Biochem Biophys Res Commun.* 384(1),126-30.
- Montecucco C., Papini E., and Schiavo G. (1994). Bacterial protein toxins penetrate cells via a four-step mechanism. *FEBS Lett.* 346(1), 92-8.
- Montecucco C. and Schiavo G. (1993). Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem Sci.* 18(9), 324-7.
- Montesino R., Garcí a R., Quintero O., and Cremata J.A. (1998). Variation in N-Linked Oligosaccharide Structures on Heterologous Proteins Secreted by the Methylophilic Yeast *Pichia pastoris*. *Protein Expression and Purification* 14, 197–207.
- Moriyama E.N. and Powell J.R. (1997) Codon Usage Bias and tRNA Abundance in *Drosophila*. *J. Mol. Evol.* 45, 514–523.
- Müller K.M., Arndt K.M., Bauer K., Plückthun A. (1998). Tandem immobilized metal-ion affinity chromatography/immunoaffinity purification of His-tagged proteins--evaluation of two anti-His-tag monoclonal antibodies. *Anal Biochem.* 259(1), 54-61.
- Müller S., Sandal T., Kamp-Hansen P., and Dalboge H. (1998). Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* 14(14), 1267-1283.

ND-1000 Spectrophotometer User's Manual V3.5 NanoDrop Technologies, Inc.  
*www.nanodrop.com*, 2007

Neill S.D., McLoughlin, M.F., and McIlroy S.G. (1989). Type C botulism in cattle being fed ensiled poultry litter. *Vet Rec.* 124(21), 558-560.

Notermans S., Dufrenne J., and Oosterom J. (1981). Persistence of *Clostridium botulinum* type B on a cattle farm after an outbreak of botulism. *Appl Environ Microbio.* 41(1), 179-183.

Ochanda J.O., Syuto B., Ohishi I., Naiki M., and Kubo K. (1986). Binding of *Clostridium botulinum* Neurotoxin to Gangliosides. *Biochem J.* 100, 27-33.

O'Leary J.M., Radcliffe C.M., Willis A.C., Dwek R.A., Rudd P.M., and Downing A.K. (2004). Identification and removal of O-linked and non-covalently linked sugars from recombinant protein produced using *Pichia pastoris*. *Protein Expression and Purification* 38(2), 217-227.

Ortolani E.L., Brito L.A., Mori C.S., Schalch U., Pacheco J., and Baldacci L. (1997). Botulism outbreak associated with poultry litter consumption in three Brazilian cattle herds. *Vet Hum Toxicol.* 39(2), 89-92.

Oshima M., Hayakari M., Middlebrook J.L., Atassi M.Z. (1997). Immune Recognition of Botulinum Neurotoxin Type A: Regions Recognized by T Cells and Antibodies Against the Protective Hc Fragment (Residues 855-1296) of the Toxin. *Molecular Immunology* 34(14), 1031-1040.

Outchkourov N.S., Stiekema W.J., and Jongsma M.A. (2002). Optimization of the expression of equistatin in *Pichia pastoris*. *Protein Expression and Purification* 24(1), 18-24.

Patra K., Mukhopadhyay R., Mukhija R., Krishnan A., Garg L.C., and Panda A.K. (2000). Optimization of Inclusion Body Solubilization and Renaturation of Recombinant Human Growth Hormone from *Escherichia coli*. *Protein Expression and Purification* 18, 182-192.

Patural H., Goffaux P., Paricio C., Emeriaud G., Teyssier G., Barthelemy J.C., Pichot V., Roche F. (2009). Infant botulism intoxication and autonomic nervous system dysfunction *Anaerobe* [Epub ahead of print].

*Pichia* Expression Kit- A Manual of Methods for Expression of Recombinant Proteins in *Pichia pastoris*. Catalog no. K1710-01 Version M. Invitrogen, 2002.

pPICZ $\alpha$  A, B, and C- *Pichia* expression vectors for selection on Zeocin and purification of secreted, recombinant proteins Catalog no. V195-20 Version E. Invitrogen, 2002.

Pla I.A., Damasceno L.M., Vannelli T., Ritter G., Batt C.A., and Shuler M.L. (2006). Evaluation of *Mut+* and *MutS* *Pichia pastoris* Phenotypes for High Level Extracellular scFv Expression under Feedback Control of the Methanol Concentration. *Biotechnol. Prog.* 22, 881-888.

Popoff M. R. and Lecoanet J. (1987). Botulinum type D toxin and *Clostridium perfringens* enterotoxin in a bull calf. *Vet Rec.* 121(25-26), 591-592.

Potter K.J., Bevins M.A., Vassilieva E.V., Chiruvolu V.R., Smith T., Smith L.A. (1998). Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification* 13(3), 357-365.

Potter K.J., Zhang W., Smith L.A., and Meagher M.M. (2000). Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification* 19(3), 393-402.

Powner D. and Davey J. (1998). Activation of the kexin from *Schizosaccharomyces pombe* requires internal cleavage of its initially cleaved prosequence. *Mol Cell Biol.* 18(1), 400-8.

Prevot A.R., Terrasse J., Daumail J., Cavaroc M., Riol J., Sillio R. (1955). Existence en France du botulisme humain de type C. *Bulletin de l'Academie de Medecine (Paris)* 139, 355-358.

QIAprep® Miniprep Handbook- For purification of molecular biology grade DNA Second Edition, December 2006. QIAGEN

Richmond J. Synaptic function ,(December 7, 2007). *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.69.1, <http://www.wormbook.org>

Romanos M.A., Makoff A.J., Fairweather N.F., Beesley K.M., Slater D.E., Rayment F.B. (1991). Expression of tetanus toxin fragment C in yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucleic Acids Res.* 19(7), 1461-1467.

Romanos M.A., Scorer C.A., and Clare J.J. (1992). Foreign gene expression in yeast: a review. *Yeast* 8, 423-488.

Rossetto O. and Montecucco C. (2007) Peculiar binding of botulinum neurotoxins. *ACS Chem Biol.* 2(2), 96-8.

Rossetto O., Seveso M., Caccin P., Schiavo G., Montecucco C. (2001). Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon* 39(1), 27-41.

Rummel A., Karnath T., Henke T., Bigalke H., Binz T. (2004). Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J Biol Chem.* 279(29), 30865-30870.

- Sakaguchi G. (1983). Clostridium botulinum toxins. *Pharmac Ther.* 19, 165-194.
- Sambrook J., Fritsch E.F., Maniatis T. (1987). Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, U.S.
- Schein C.H. (1989). Production of soluble recombinant proteins in bacteria. *Biotechnology (NY)* 7, 1141-1149.
- Schroeter M., Alpers K., Van Treeck U., Frank C., Rosenkoetter N., And Schaumann R. (2009). Outbreak of wound botulism in injecting drug users. *Epidemiol Infect.* [Epub ahead of print].
- Schwarz K., Fiedler T., Fischer R.J., Bahl H. (2007). A Standard Operating Procedure (SOP) for the preparation of intra- and extracellular proteins of *Clostridium acetobutylicum* for proteome analysis. *Journal of Microbiological Methods* 68, 396–402.
- Shakin-Eshleman S.H., Spitalnik S.L. and Kasturi L. (1996). The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *J Biol Chem.* 271(11), 6363-6366.
- Shams H. (2005). Recent developments in veterinary vaccinology. *Vet J.* 170(3), 289-299.
- Shapiro A.L., Viñuela E. and Maizel J.V. Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun.* 28(5), 815-20.
- Shepard S.R., Boucher R., Johnston J., Boerner R., Koch G., Madsen J.W., Grella D., Sim B.K., Schrimsher J.L. (2000). Large-Scale Purification of Recombinant Human Angiostatin. *Protein Expression and Purification* 20(2), 216-227.
- Shiningavamwe A., Obiero G., Albertyn J., Nicaud J.M., Smit M. (2006). Heterologous expression of the benzoate para-hydroxylase encoding gene (CYP53B1) from *Rhodotorula minuta* by *Yarrowia lipolytica*. *Appl Microbiol Biotechnol.* 72, 323–329.
- Shukla H.D. and Sharma S.K. (2005). *Clostridium botulinum*: a bug with beauty and weapon. *Crit Rev Microbiol.* 31(1), 11-18.
- Simpson L.L. and Rapport M.M. (1971). Ganglioside inactivation of botulinum toxin. *Journal of Neurochemistry* 18, 1341-1343.
- Singh S., Gras A., Fiez-Vandal C., Ruprecht J., Rana R., Martinez M., Strange P.G., Wagner R., Byrne B. (2008). Large-scale functional expression of WT and truncated human adenosine A2A receptor in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories* 7:28.
- Singh M. and O'Hagan D.T (2003). Recent advances in veterinary vaccine adjuvants. *International Journal for Parasitology* 33,469–478.

- Smith L.A. (1998). Development of recombinant vaccines for botulinum neurotoxin. *Toxicon* 36(11), 1539-1548.
- Sobel J. (2005). Botulism. *Clin Infect Dis.* 41(8), 1167-1173.
- Sreekrishna K., Brankamp R.G., Kropp K.E., Blankenship D.T., Tsay J.T., Smith P.L. (1997). Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 190(1), 55-62.
- Steinman A., Chaffer M., Elad D., Shpigel N.Y. (2006). Quantitative analysis of levels of serum immunoglobulin G against botulinum neurotoxin type D and association with protection in natural outbreaks of cattle botulism. *Clin Vaccine Immunol.* 13(8), 862-8.
- Steinman A., Galon N.B., Arazi A., Bar-Giora Y., Shpigel N.Y. (2007). A Cattle immune response to botulinum type D toxoid: Results of a vaccination study. *Vaccine* 25(44), 7636–7640.
- Stratford R., Douce G., Zhang-Barber L., Fairweather N., Eskola J. Dougan G. (2001). Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 19(7-8), 810–815.
- Sugiyama H. (1980). *Clostridium botulinum* neurotoxin. *Microbiol Rev.* 44(3), 419-448.
- Sun H.X., Xie Y., Ye Y.P. (2009). Advances in saponin-based adjuvants. *Vaccine* 27(12), 1787–1796.
- Sunagawa H., Ohyama T., Watanabe T., Inoue K. (1992). The complete amino acid sequence of the *Clostridium botulinum* type D neurotoxin, deduced by nucleotide sequence analysis of the encoding phage d-16 phi genome. *J. Vet. Med. Sci.* 54(5), 905-913.
- Syuto B. and Kubo S. (1981). Separation and characterization of heavy and light chains from *Clostridium botulinum* type C toxin and their reconstitution. *J Biol Chem.* 256(8), 3712-3717.
- Te'o V.S.J., Cziferszky A.E., Bergquist P.L., Nevalainen K.M.H. (2000). Codon optimization of xylanase gene *xynB* from the thermophilic bacterium *Dictyoglomus thermophilum* for expression in the filamentous fungus *Trichoderma reesei*. *FEMS Microbiology Letters* 190, 13-19.
- Thiry M. and Cingolani D. (2002). Optimizing scale-up fermentation processes. *Trends in Biotechnology* 20(3). 103-105.
- Thomas R.J. (1991). Detection of *Clostridium botulinum* types C and D toxin by ELISA. *Aust Vet J.* 68(3), 111-113.
- Ting P.T. and Freiman A. (2004). The story of *Clostridium botulinum*: from food poisoning to Botox. *Clin Med.* 4(3), 258-261.

Towbin H., Staehelin T., and Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci.* 76(9), 4350-4354.

Trinh L.B., Phue J.N., Shiloach J. (2003). Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. *Biotechnol Bioeng.* 82(4), 438-444.

Trueman, K. F., Bock, R. E., Thomas, R. J., Taylor, J. D., Green, P. A., Roeger, H. M., Ketterer P.J. (1992). Suspected botulism in three intensively managed Australian cattle herds. *Vet Rec.* 130(18), 398-400.

Tsuzuki K. , Yokosawa N. , Syuto B. , Ohishi I. , Fujii N. , Kimura K. , Oguma K (1988). Establishment of a monoclonal antibody recognizing an antigenic site common to *Clostridium botulinum* type B, C1, D, and E toxins and tetanus toxin. *Infect Immun.* 56(4), 898-902.

Turner L. and Stephens J. (2008). Vaccinating dairy cattle against disease. Economic considerations. *DPI&F Note*, Queensland Government found in site: <http://www2.dpi.qld.gov.au/dairy/13252.html>

Turton K., Chaddock J.A., and Acharya K.R. (2002). Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *Trends Biochem Sci.* 27(11), 552-558.

Vassileva A., Chugh D.A., Swaminathan S., and Khanna N. (2001). Effect of Copy Number on the Expression Levels of Hepatitis B Surface Antigen in the Methylotrophic Yeast *Pichia pastoris*. *Protein Expression and Purification* 21, 71-80.

Viader-Salvadó J.M., Cab-Barrera E.L., Galán-Wong L.J., Guerrero-Olazarán M. (2006). Genotyping of recombinant *Pichia pastoris* strains. *Cellular & Molecular Biology Letters* 11, 348-359.

Vordermeier M. and Hogarth P. (2005). Vaccine development in the 21st century: a time of living dangerously. *Vet J.* 170(3), 271-272.

Webb R.P., Smith T.J., Wright P.M., Montgomery V.A., Meagher M.M., Smith L.A. (2007). Protection with recombinant *Clostridium botulinum* C1 and D binding domain subunit (Hc) vaccines against C and D neurotoxins. *Vaccine* 25(21), 4273-4282.

Whalen R.L., Dempsey D.J., Thompson L.M., Bucknell K., Kunitomo R., Okazaki Y., Harasaki H. (1996). Microencapsulated vaccines to provide prolonged immunity with a single administration. *ASAIO J.* 42(5), 649-654.

Whittaker M.M. and Whittaker J.W. (2000). Expression of recombinant galactose oxidase by *Pichia pastoris*. *Protein Expression and Purification* 20(1),105-11.



- Woo J.H., Liu Y.Y., Mathias A., Stavrou S., Wang Z., Thompson J., Neville D.M. (2002). Gene optimization is necessary to express a bivalent anti-human anti-T cell immunotoxin in *Pichia pastoris*. *Protein Expression and Purification* 25, 270–282.
- Woodward L.A., Arimitsu H., Hirst R., and Oguma K. (2003). Expression of HC subunits from *Clostridium botulinum* types C and D and their evaluation as candidate vaccine antigens in mice. *Infect Immun.* 71(5), 2941-2944.
- Wu H., Singh N.K., Locy R.D., Scissum-Gunn K., Giambrone J.J. (2004). Immunization of chickens with VP2 protein of infectious bursal disease virus expressed in *Arabidopsis thaliana*. *Avian Diseases* 48, 663-668.
- Xie J., Zhang L., Ye Q., Zhou Q., Xin L., Du P., Gan R. (2003). Angiostatin production in cultivation of *Pichia pastoris* fed with mixed carbon sources. *Biotechnol Letts.* 25, 173–177.
- Yan B., Zhang W., Ding J. and Gao P. (1999). Sequence Pattern for the Occurrence of N-Glycosylation in Proteins. *Journal of Protein Chemistry* 18, 5511-521.
- Yonekura-Sakakibara K. and Saito K. (2006). Review: genetically modified plants for the promotion of human health. *Biotechnol Lett* 28, 1983–1991.
- Yowler B.C. and Schengrund C.L. (2004). Glycosphingolipids-sweets for botulinum neurotoxin. *Glycoconjugate Journal* 21, 287–293.
- Yu X., Li Z., Xia X., Fang H., Zhou C., Chen, H. (2007). Expression and purification of anicrod, an anticoagulant drug, in *Pichia pastoris*. *Protein Expression and Purification* 55(2), 257-261.
- Yun-Zhou Y., Na L., Heng-Qi Z., Rui-Lin W., Yun D., Shuang W., Wei-Yuan Y., Zhi-Wei S. (2009). The recombinant Hc subunit of *Clostridium botulinum* neurotoxin serotype A is an effective botulism vaccine candidate. *Vaccine* 27(21), 2816–2822.
- Zdanovsky A.G. and Zdanovskaia M.V. (2000). Simple and efficient method for heterologous expression of clostridial proteins. *Appl Environ Microbiol.* 66(8), 3166-3173.
- Zhou Y. and Singh B.R. (2004). Cloning, high-level expression, single-step purification, and binding activity of His6-tagged recombinant type B botulinum neurotoxin heavy chain transmembrane and binding domain. *Protein Expression and Purification* 34(1), 8-16.

**Appendix I - Alignment of nucleotide sequences of BoNT/C and BoNT/D subunits as provided by Epoch Biolabs.**

```

      10      20      30      40      50      60
BoNT/C CCCTTTAATATTTTTTTCATATACTAATAATTCTTTATTAAAAGATATAATTAATGAATAT
      :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BoNT/D CCTTTTAATATTTTTTTCATATACTAATAATTCTTTATTAAAAGATATAATTAATGAATAT
      10      20      30      40      50      60

      70      80      90      100     110     120
BoNT/C TTCAATAATATTAAT-ATTCAAAAATTTTGAGCCTACAAAACAGAAAAAATACTTTAGTG
      ::::: ::::: :::::::::::::::::::: ::::: ::::: ::::: :::::
BoNT/D TTCAATAGTATTAATGATTCAAAAAATTTTGAGCTTACAAAACAAAAAATGCTTTAGTG
      70      80      90      100     110     120

      130     140     150     160     170     180
BoNT/C GATACATCAGGATATAATGCAGAAGTGAGTGAAGAAGGCGATGTTTCAGCTTAATCCAATA
      ::::::::::::::::::::::::::::::: : :: : : ::::: ::::: : ::
BoNT/D GATACATCAGGATATAATGCAGAAGTGAGGGTAGGAGATAATGTTCAACTTAATACGATA
      130     140     150     160     170     180

      190     200     210     220     230     240
BoNT/C TTTCCATTTGACTTTAAATTAGGTAGTTCAGGGGAGGATAGAGGTAAAGTTATAGTAACC
      : : :: :::::::::::::::::::: ::::: ::::: :::::
BoNT/D TATACAAATGACTTTAAATTAAGTAGTTCAGGAGA-----TAAAATTATAGTAAAT
      190     200     210     220     230

```

250          260          270          280          290          300  
 BoNT/C CAGAATGAAAATATTGTATATAAATCTATGTATGAAAGTTTTAGCATTAGTTTTTTGGATT  
           ::: : ::::: :::::        ::: :::: : : ::: ::::: :::::  
 BoNT/D TTAAATAATAATTTTTATATAGCGCTATTTATGAGAACTCTAGTGTTAGTTTTTTGGATT  
           240          250          260          270          280          290  
  
 310          320          330          340          350          360  
 BoNT/C AGAATAAATAAATGGGTAAGTAATTTACCT---GGATATACTATAAATTGATAGTGTTAAA  
           : ::: ::::        ::: ::::: : : : ::::: ::::: : ::: : ::  
 BoNT/D AAGATATCTAAAGATTTAACTAATTTCTCATAATGAATATACAATAATTAACAGTATAGAA  
           300          310          320          330          340          350  
  
 370          380          390          400          410          420  
 BoNT/C AATAACTCAGGTTGGAGTATAGGTATTATTAGTAATTTTTTAGTATTTACTTTAAAACAA  
           : : :: : : ::::        :: ::::: : : : : ::: : : : ::::: : :  
 BoNT/D CAAAATTCTGGGTGGAAATTATGTATTAGGAATGGCAATATAGAATGGATTTTACAAGAT  
           360          370          380          390          400          410  
  
 430          440          450          460          470          480  
 BoNT/C AATGA-AGATAGTGAACAAAGTATAAAATTTTAGTTATGATATATCAAATAATGCTCCTGG  
           : : :::: : : : ::::: ::: :::: : :::: : : :::: : : : :::  
 BoNT/D GTTAATAGAAAGTATA-AAAGTTTAATTTTTGATTATAGTGAATCATTAAAGTCATACAGG  
           420          430          440          450          460          470

```

490      500      510      520      530      540
BoNT/C ATACA---ATAAATGGTTTTTTTGTAAGTTACTACTAACAATATGATGGGAAATATGAAGAT
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
BoNT/D ATATACAAATAAATGGTTTTTTTGTTACTATAACTAATAATATAATGGGGTATATGAAACT
      480      490      500      510      520      530

      550      560      570      580      590      600
BoNT/C TTATATAAATGGAAAATTAATAGATACTATAAAAAGTTAAAGAACTAACTGGAATTAATTT
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
BoNT/D TTATATAAATGGAGAATTAAAGCAGAGTCAAAAAATTTGAAGATTTAGATGAGGTTAAGTT
      540      550      560      570      580      590

      610      620      630      640      650      660
BoNT/C TAGCAAAACTATAACATTTGAAATAAATAAAAATTCCAGATACCGGTTTGATTACTTCAGA
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
BoNT/D AGATAAAACCATAGTATTTGGAATAGATGAGAATATAGAT-----GAGAA--TCAGA
      600      610      620      630          640

      670      680      690      700      710      720
BoNT/C TTCTGATAACATCAATATGTGGATAAGAGATTTTTTATATATTTGCTAAAGAATTAGATGG
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
BoNT/D TGCTT-----TGGATTAGAGATTTTAATATTTTTTCTAAAGAATTAAGTAA
      650      660      670      680

      730      740      750      760      770      780
BoNT/C TAAAGATATTAATATATATTATTTAATAGCTTGCAATATACTAATGTTGTAAAAGATTATTG
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
      :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
BoNT/D TGAAGATATTAATATTGTATATGAGGGACAAATATTAAGAAATGTTATTAAAGATTATTG
      690      700      710      720      730      740

```

790 800 810 820 830 840  
BoNT/C GGGAAATGATTTAAGATATAATAAAGAATATTATATGGTTAATATAGATTATTTAAATAG

: :

BoNT/D GGGAAATCCTTTGAAGTTTGATACAGAATATTATATTTAATGATAATTATATAGATAG  
750 760 770 780 790 800

850 860 870 880 890

BoNT/C ATATAT-GTATGCGAAC-TCACGACAAATTGTTTTTAATACACGTAGAAAATAATAATGAC  
: :

BoNT/D GTATATTGCACCTGAAAGTAATG--TACTTGTACTTGTTCGGTATCCAGATAGATCTAAA  
810 820 830 840 850 860

900 910 920 930 940 950

BoNT/C TTCAATGAAGGATATAAAAATTATAATAAAA--AGAATCAGAGGAAATACAAATGATACTA  
: :

BoNT/D TTATATACTGGAAATCCTATTACTATTAAATCAGTATCTGA--TAAGAATCCTTATAGTA  
870 880 890 900 910 920

960 970 980 990 1000 1010

BoNT/C GAGTACGAGGAGGAGATATTTTATATTTTGATATGACAATTAATAACAAAGCATATAAAT  
: :

BoNT/D GAATTTTAAATGGAGATAATATAAATTCATATGTTATATAATAGTAGGAAATATATGA  
930 940 950 960 970 980

1020 1030 1040 1050 1060 1070

BoNT/C TGTTTATGAAGAATGAAACTATGTATGCAG-ATAATCATAGTACTGAAGATATATATGC-  
: :

BoNT/D TAATAAGAGATACTGATACAATATATGCAACACAAGGAGGAGAGTGTTTCACAAAATTGTG  
990 1000 1010 1020 1030 1040

```

1080      1090      1100      1110      1120      1130
BoNT/C TATAGGT-TTAAGAGAACAAACAAAGGATATAAATGATAATAT---TATATTTCAAATAC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BoNT/D TATATGCATTAAAATTACAGAGTAATTTAGGTAATTATGGTATAGGTATATTTAGTATAA
1050      1060      1070      1080      1090      1100
1140      1150      1160      1170      1180      1190
BoNT/C AACCAATGAATAATACTTATTATTACGCATCTCAAATATTTAAATCAAATTTTAATGGAG
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BoNT/D AAAATATTGTATCTAAAAATAAATATTGTAGTCAAATTTTC---TCTAGTTTTAG-GGAA
1110      1120      1130      1140      1150
1200      1210      1220      1230      1240      1250
BoNT/C AAAATATTTCTGGAATATGTTCAATAGGTACTTATCGTTTTAGACTTGGAGGTGATTG-G
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BoNT/D AATACAATGCTTCTAGCAGATATATA-----TAAACCTTGGAGATTTTCTTTTAAAAATG
1160      1170      1180      1190      1200      1210
1260      1270      1280      1290      1300
BoNT/C TATAGACACAATTATTTGGTGCCTACTGTGAAGCAAGGAAATTATG--CTTCAT--TATT
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BoNT/D CATAACGCCAGTTGCAGTAACTAATTATGAAACAAAACCTATTATCAACTTCATCTTTTTT
1220      1230      1240      1250      1260      1270
1310      1320      1330      1340      1350      1360
BoNT/C AGAA-TCAACATCAACTCATTGGGGTTTTGTACCTGTAAGTGAATAAATAATGATTAATA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BoNT/D GGAAATTTATTTCTAGGGATCCAGGATGGGTAGA-GTAATACAATAAAAAATTTAATATAA
1280      1290      1300      1310      1320      1330

```

	1370	1380	1390	1400
BoNT/C	A-TAT-AAATTATGTTAAATATTTTAATA--TTAAGTTGAAATATGTT			
	:	:::	:::::	:::
	:	:	:	:
	:	:	:	:
	:	:	:	:
BoNT/D	ACTATTAAATTATATTACAAGTTTTAGAAATTTATCGTATAAAATGTT			
	1340	1350	1360	1370
				1380

## Appendix II - Amino acid sequences of the original, unmodified BoNT/C and BoNT/D subunits obtained from Dr. Woodward

### BoNT/C Original

Y S K I L S L Q N R K N T L V D T S G Y N A E V S E E G D V Q L N P I F P F D F K  
L G S S G E D R G K V I V T Q N E N I V Y N S M Y E S F S I S F W I R I N K W V S N  
L P G Y T I I D S V K N N S G W S I G I I S N F L V F T L K Q N E D S E Q S I N F S Y  
D I S N N A P G Y N K W F F V T V T N N M M G N M K I Y I N G K L I D T I K V K  
E L T G I N F S K T I T F E I N K I P D T G L I T S D S D N I N M W I R D F Y I F A K  
E L D G K D I N I L F N S L Q Y T N V V K D Y W G N D L R Y N K E Y Y M V N I D  
Y L N R Y M Y A N S R Q I V F N T R R N N N D F N E G Y K I I I K R I R G N T N D  
T R V R G G D I L Y F D M T I N N K A Y N L F M K N E T M Y A D N H S T E D I Y  
A I G L R E Q T K D I N D N I I F Q I Q P M N N T Y Y Y A S Q I F K S N F N G E N I  
S G I C S I G T Y R F R L G G D W Y R H N Y L V P T V K Q G N Y A S L L E S T S T  
H W G F V P V S E Stop

### BoNT/D Original

P F N I F S Y T N N S L L K D I I N E Y F N S I N D S K I L S L Q N K K N A L V D T S  
G Y N A E V R V G D N V Q L N T I Y T N D F K L S S S G D K I I V N L N N N I L Y  
S A I Y E N S S V S F W I K I S K D L T N S H N E Y T I I N S I E Q N S G W K L C I R  
N G N I E W I L Q D V N R K Y K S L I F D Y S E S L S H T G Y T N K W F F V T I T  
N N I M G Y M K L Y I N G E L K Q S Q K I E D L D E V K L D K T I V F G I D E N I  
D E N Q M L W I R D F N I F S K E L S N E D I N I V Y E G Q I L R N V I K D Y W G  
N P L K F D T E Y Y I I N D N Y I D R Y I A P E S N V L V L V R Y P D R S K L Y T G  
N P I T I K S V S D K N P Y S R I L N G D N I I L H M L Y N S R K Y M I I R D T D T I  
Y A T Q G G E C S Q N C V Y A L K L Q S N L G N Y G I G I F S I K N I V S K N K Y  
C S Q I F S S F R E N T M L L A D I Y K P W R F S F K N A Y T P V A V T N Y E T K  
L L S T S S F W K F I S R D P G W V E Stop



**Appendix III - Raw sequencing data of subunits BoNT/C and BoNT/D from Macrogen, Korea. See section 3.2 for information regarding sequencing primers used**

BoNT/C – forward sequence

GGTTGAAGAGGATCCTCGGAAGAGAGGCTGAGCTGAATTCTGGAGAACC  
TATTCCTTTCAACATCTTCTCCTACACCCATAACTCTTTGTTGAAGGACA  
TCATTAACGAGTACTTCAACAACATCCACGACTCCAAGATTTTGTCTTG  
CAAACCGTAAGAACACCTTGGTTGATACCTCTGGTTACAACGCTGAAGT  
CTCCGAGGAGGGTGATGTTCAATTGAACCAATCTTCCCTTTCGACTTCA  
AGTTGGGTTCTTCCGGTGAGGACAGAGGAAAGGTCATTGTCACTCAAAC  
GAAAACATCGTTTACAACCTCCATGTACGAATCTTCTCTATCTCCTTCTG  
GATTAGAATCAACAAGTGGGTTTCCAACCTTGCCAGGATACACCATCATCG  
ACTCTGTTAAGAACAACCTCCAGTTGGTCTATCGGAATCATCTCTAACTTC  
TTGGTTTTCACTTTGAAGCACAAACGAGGATTCTGAACAATCTATCCACTT  
CTCCTAAGATATCTCCAACAACGCTCCAGGTTACAACAAGTGGTTCTTCG  
CCACTGTTACCAACATCATGATGGGTAACATGAAGATCTACATCAACGGA  
AAATTGATCGACACCATTAAGGATAAGGAGTTGACCGGTATCCACTTCTC  
TAAGACCATCTCCTTCGAAATTAACAAGATCCCAGACACCGGTTTGATTA  
CCTCCGATTCTGACAACATTAACATGAGTATTAGAGACTTCTACATCTTC  
GCTAACGAGTTGGACGGTAAAGACATCTACATCTTGTTCAACTCTTTGCA  
ATACACTAACGTTGCTTAGGACTACTGGGGTAACGATTTGAGCTACCACA  
TGGAGTCCTCCATGGTCTAACATTGACTACTAGAATCGTTTCATGTCCGC  
TCACTTCAAAAAATCCTCTTCATATTCC

BoNT/C – reverse sequence

AAGTAATCAGACGGTCTTCCGTAGTGCCCACTTGAACTGAGGAACA  
GTCATGTCTAAGGCTACAAACTCAATGATGATGATGATGATGGTCGACGG  
CGCTATTCAGATCCTCTTCTGAGATGAGTTTTTGTCTAGACCTTCAGAA  
ACTGGAACGAAACCCCAATGAGTAGAAGTAGATTTCGAGGAGAGAAGCGTA  
GTTACCCTGCTTAACAGTTGGGACGAGGTAGTTATGACGGTACCAGTCAC  
CTCCCAAACGGAATCTGTAAGTACCGATGGAACAGATAACCAGAGATGTTT  
TCACCGTTGAAGTTGGATTTGAAGATCTGAGAAGCGTAGTAGTAAGTGTT  
GTTCAATTGGCTGGATCTGGAAAATGATGTTGTCGTTGATGTCCTTAGTTT  
GTTCTCTCAAACCGATGGCGTAGATGTCTTCGGTGGAGTGGTTGTCAGCG  
TACATGGTCTCGTTCTTCATGAACAAGTTGTAGGCCTTGTTGTTGATAGT  
CATGTCGAAGTACAAGATGTCTCCACCTCTGACTCTGGTATCATGAGTGT  
TACCTCTGATACGCTTGATAATGATCTTGTAACCCTCGTTGAAGTCGTTG  
TTGTTTCTACGAGTGTTGAAGACGATTTGTCTGGAGTTGGCGTACATGTA  
ACGGTTCAAGTAGTCAATGTTGACCATGTAGTACTCCTTGTTGTATCTCA  
AATCGTTACCCAGTAGTCCTTAACAACGTTAGTGTATTGCAAAGAGTTG  
AACAAGATGTTGATGTCTTTACCGTCCAACCTCCTTAGCGAAGATGTAGAA  
GTCTCTAATCCACATGTTAATGTTGTCAGAATCGGAGGGTATCAAACCGG  
TGTCTGGGATCTTGTTAATTTCNAAAGTGATGGTCTTAGAGAAGTGGATA  
CCGGTCAACTCCTTAACCTTAATGGGGTCGATCCATTTTCCGTGGAGGGG  
AATCTCATGTTACCCACCTGTTGT

BoNT/D – forward sequence

GGATGAAAATGGATCTTGAGAAGAAGGCTGAGCTGAATTCTTTAATAT  
GTTTCAGTTATACGCATAACTCCTTGTTGAAAGATATTATCAACGAGTACT  
TCAACAGCATTTCATGATAGCAAGATTTTGAGCTTGCAGAACAAAAAGAAT  
GCTCTAGTTGATACCAGTGGATACAACGCTGAAGTGAGGGTCGGTGATAA  
TGTGCAACTGAACACTATCTATACGAATGACTTCAAGCTATCCTCATCCG  
GCGATAAAATCATTGTCAACCTAAATAATAACATTCTGTACTCCGCTATA  
TACGAACACTCAAGTGTGTCTTTTTGGATCAAGATATCTAAAGATTTAAC  
TAATTCCCACAACGAGTATACTATAATCAATAGTATTGAACAGAACTCTG  
GTTGGAAGTTGTGCATTAGGAATGGCAACATCGAATGGATTTTACAGGAT  
GTCAACCGTAAATATAAGTCTCTAATTTTTGATTACTCCGAGTCATTGAG  
TCATACGGGCTATACGAATAAATGGTTCTTTGTCACCATTACAAATAACA  
TAATGGGTTACATGAAATTGTACATCAATGGCGAATTGAAGCAATCCCAA  
AAGATAGAAGATTTAGATGAGGTCAAGTTGGACAAGACAATTGTTTTTGG  
AATCGACGAGAATATTGACGAAAATCAGATGTTGTGGATTAGAGATTTTA  
ATATATTTTCAAAGAATTGTCCAATGAAGATATCAACATCGTTTACGAA  
GGTCAAATCTTGAGAAATGTTATAAAGGACTATTGGGGTAACCCGTTGAA  
ATTTGACACTGAGTATTATATCATTAATGACCACTACATCGATAGATAACA  
TCGCACCTGAATCCATGTGTTGGTCTTGGTGAGATATCCGGACCGTTCTA  
AGTTATATACTGGCAATCCCATCACTATAAATCTGTTTCTGACAAAAACC

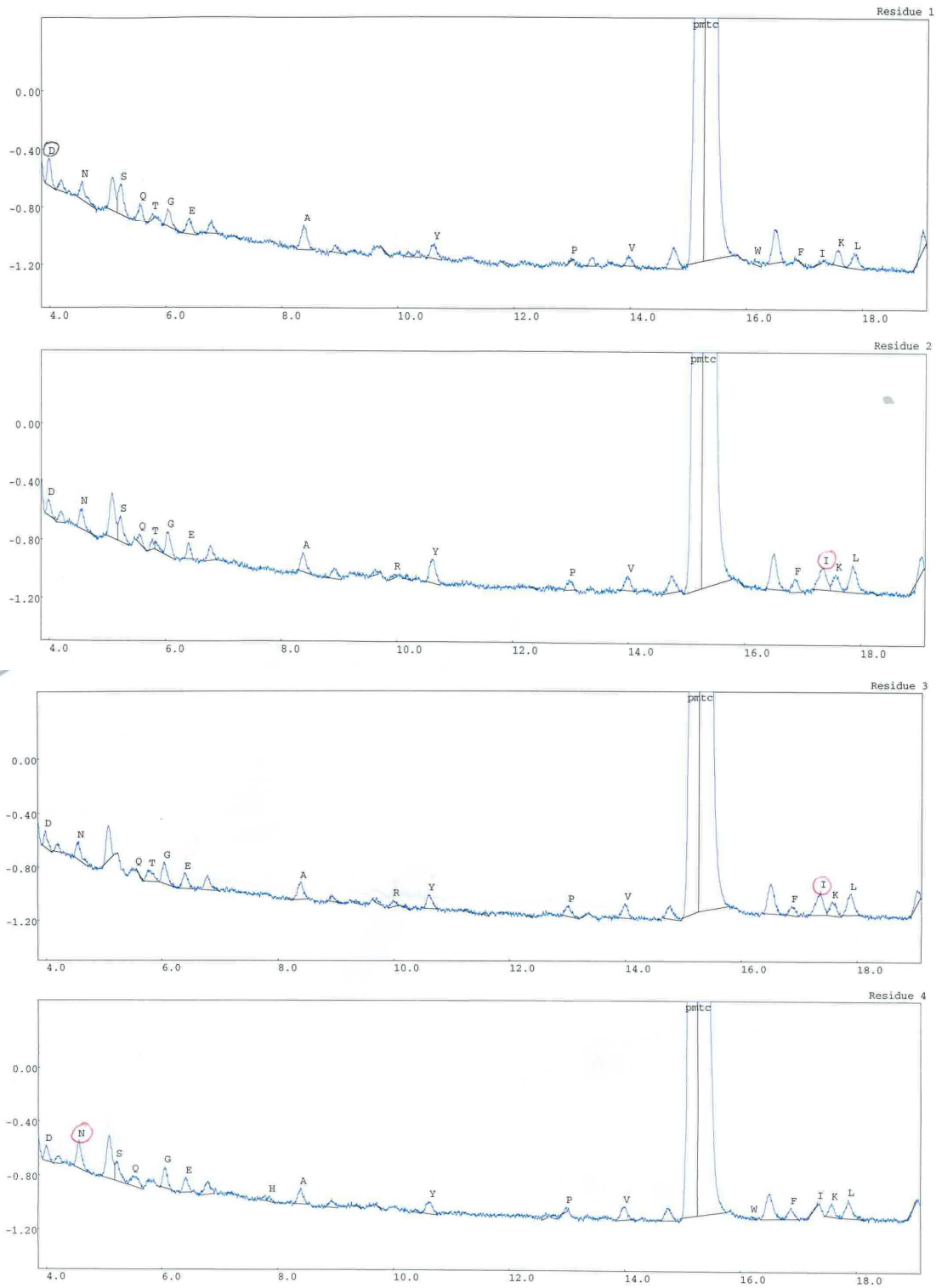
BoNT/D – reverse sequence

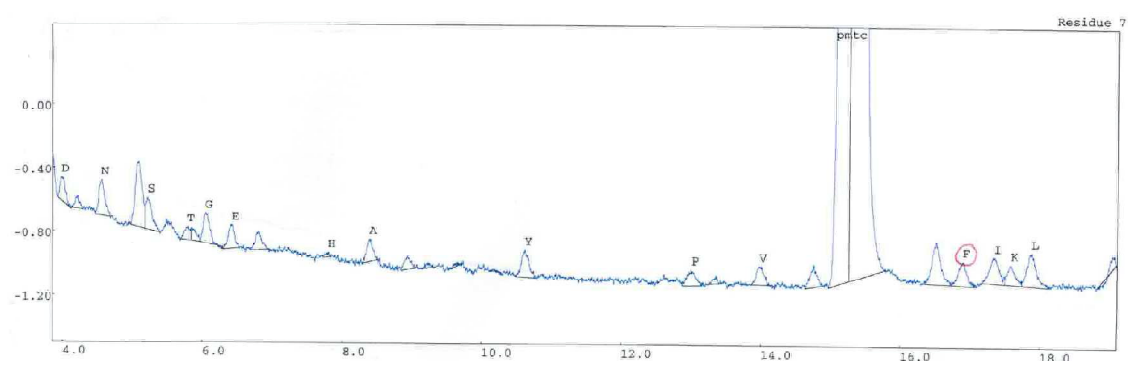
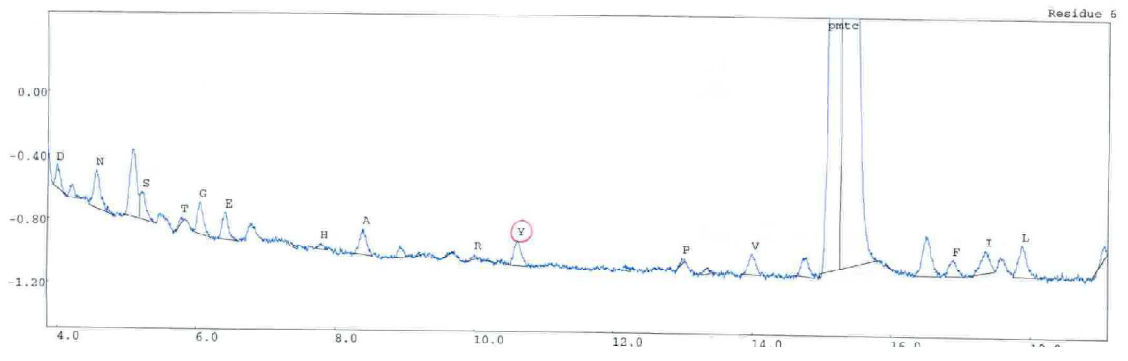
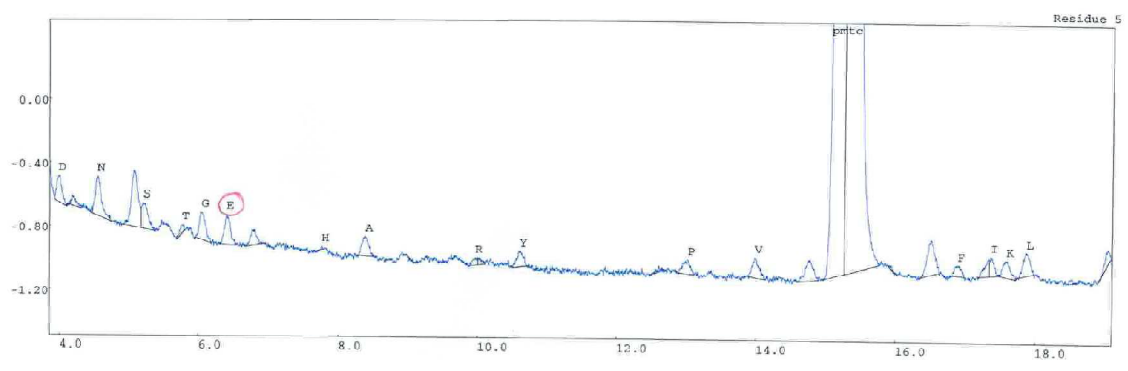
GGTACACAGACGGTCTCTCGTAGTGCCCACTTGAAGTGAAGG  
AACAGTCATGTCTAAGGCTACAACTCAATGATGATGATGATGATGGTCG  
ACGGCGCTATTTCAGATCCTCTTCTGAGATGAGTTTTTGTCTAGACCTTC  
CACCCATCCGGGATCCCTTGAAATAAACTTCCAGAAAGAGGAAGTAGACA  
GTAGCTTCGTTTCATAATTAGTCCAGCCACAGGGGTATAAGCATTCTTA  
AAAGAAAATCTCCAAGGCTTGTAGATGTCAGCCAGCAGCATAGTGTTC  
CCTGAAGGAGCTAAAAATTTGACTACAGTATTTGTTTTTGGAAACAATAT  
TCTTAATAGAAAATATTCCAATACCATAGTTACCCAGGTTTACTGCAAT  
TTTAATGCGTAAACACAGTTTTGAGAACACTCTCCACCCTGAGTGGCGTA  
GATTGTGTGCGGTATCTCTGATTATCATGTATTTCTGCTGTTGTACAGCA  
TATGCAAGATGATGTTATCTCCGTTTAAGATTCTACTGTATGGGTTTTTG  
TCAGAAACAGATTTTATAGTGATGGGATTGCCAGTATATAACTTATAACG  
GTCCGGATATCTCACCAAGACCAACACATTGGATTCAGGTGCGATGTATC  
TATCGATGTAGTTGTCATTAATGATATAATACTCAGTGTCAAATTGCAAC  
GGGTTACCCCAATAGTCCTTTATAACATTTCTCCGGATTTGACCTTCGTA  
AACGATGTTGATATCTTCATTGGACAATTCTTTTGGAAATTATTAATAATC  
TCTAATCCACATCATGTGATTTACGTCCCTATATCGTCGAATCCAAACCA  
AAGGCTTGCCCACTTGAATCTGTTAATCATCTACTATTGGGATGCTACAG  
ACGCAAAAATGAACGGTTCTGGCACGCACGTGTCATTGTAAGGGAACAAG  
AACCAATTACCAACGCCCGTTGAGCATGACTCGATAA

## Appendix IV - Multiple cloning site, KEX2 cleavage site $\alpha$ -factor and polyhistidine tag of pPICZ $\alpha$ A expression vector (Invitrogen)

5' end of AOX1 mRNA  
 5' AOX1 priming site  
 811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA  
 871 CAAGCTTTTG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT  
 931 ATTCGAAACG **ATG** AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA  
 Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala  
 983 TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA  
 Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala  
 $\alpha$ -factor signal sequence  
 1034 CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC  
 Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe  
 1085 GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT  
 Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe  
 1136 ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC  
 Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu  
 Xho I\*  
 1187 GAG AAA AGA GAG GCT GAA GCT GAATTCAC GTGGCCAG CCGGCCGTC TCGGATCGGT  
 Glu Lys Arg Glu Ala Glu Ala  
 Kex2 signal cleavage EcoR I Pml I Sfi I BsmB I Asp718  
 Ste13 signal cleavage  
 Kpn I Xho I Sac II Not I Xba I c-myc epitope  
 1244 ACCTCGAGCC GCGGCGGCC GCCAGCTTTC TA GAA CAA AAA CTC ATC TCA GAA GAG  
 Glu Gln Lys Leu Ile Ser Glu Glu  
 polyhistidine tag  
 1299 GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTGTAGCC  
 Asp Leu Asn Ser Ala Val Asp His His His His His His \*\*\*  
 1351 TTAGACATGA CTGTTCTCA GTTCAAGTTG GGCACCTACG AGAAGACCGG TCTTGCTAGA  
 3' AOX1 priming site  
 1411 TTCTAATCAA GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATT TTTGAT  
 3' polyadenylation site  
 1471 ACTTTTTTAT TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT

## Appendix V - Protein sequencing data for BoNT/C subunit, performed by the University of Newcastle, NSW.





## Appendix VII - Certificate of analysis of *C. botulinum* toxins ordered from Metabiologics, INC

### METABIOLOGICS, INC

505 S. ROSA RD, SUITE 18  
MADISON WI 53719  
(608) 441-2730 fax (608) 441-2731

### Certificate of Analysis

Description: Clostridium botulinum Type C & D Neurotoxin (Purified)

Lot Number, Quantity, Volume, Specific Activity and concentration of NT:

D110807-01 0.2 mg 0.2 ml  $9 \times 10^7$  LD50/mg 1.0 mg/ml

C110807-01 0.2 mg 0.2 ml  $1.9 \times 10^7$  LD50/mg 1.0 mg/ml

The Neurotoxins (NT) were shipped in PBS, pH 7.0 and have been 0.22 micron filtered.

The neurotoxins were tested for identity by neutralization with type specific antibodies. Activity was determined by mouse bioassay. Purity was checked by SDS-PAGE.

Storage conditions: Refrigerate (2-8 C) or Freeze (Long term).