New High Affinity Antibodies Against Botulinum Neurotoxin Type A

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New high affinity antibodies against Botulinum Neurotoxin Type A have been developed in chickens. Production of polyclonal IgY is both easy and cost effective. A single egg contains as much antibody as a single bleed from a rabbit and is a simple and less invasive production method. Eggs from immunized chickens represent a continual source of polyclonal antibody.

The antigen used to challenge the chicken was a recombinant Heavy Chain Binding domain fragment (HccA) from Type A toxin. IgY derived from eggs were affinity purified using an antigen-coated column. To test the affinity purified antibodies we determined the titer of the anti-HccA antibodies against Botulinum Neurotoxin Type A (BTA) holotoxin, List Product #130. The ability of the anti-HccA antibodies to capture BTA was also tested using a "Sandwich" ELISA.

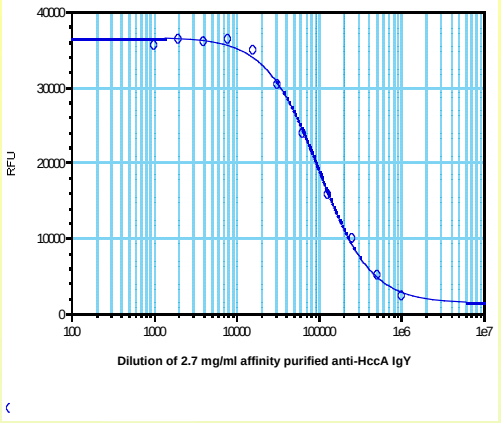

FIGURE 1: Titer determination –"Indirect" ELISA
Two micrograms of BTA was coated per well of a 96 well microtiter plate. Purified IgY stock at 2.7 mg/ml was titrated by 2 fold serial dilutions across the plate. A rabbit derived anti-IgY HRP conjugate was used as the secondary antibody at 1:30,000. QuantaBlu peroxidase fluorescent substrate from Pierce was used to develop the plate. The "Indirect " ELISA measured the antibody titer of IgY against 2 µg BTA to be approximately 1:100,000.

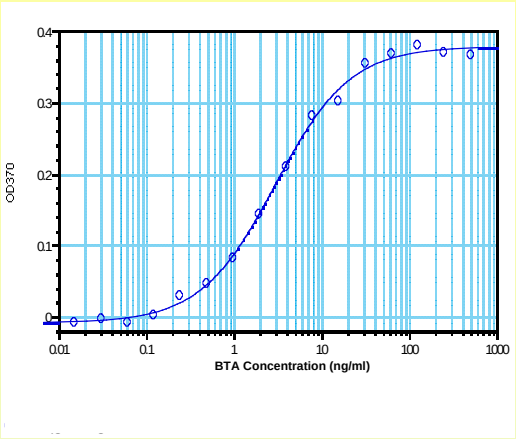

FIGURE 2: Detection of BTA – "Sandwich" ELISA
To determine the ability of our new IgY antibody to capture and detect BTA we used a Sandwich ELISA to determine detection sensitivity. 2 µg of IgY was coated per well of a 96 well microtiter plate. BTA was titrated across the plate and allowed to bind to the IgY. An anti-BTA horse derived antibody and a rabbit derived anti-Horse HRP antibody were used at 1:1000 and 1:2000 respectively, to detect the binding of BTA to IgY. 1-Step Turbo TMB from Pierce was used as peroxide substrate to develop. The midpoint of the detection curve is 2.9 ng/ml and sample as low as 0.244 ng/ml can be detected above background.

Comparison of FRET Substrates for Botulinum Neurotoxin Type A

A new FRET substrate for the zinc endoprotease activity of the 50 kDa light chain (LcA) of the type A Botulinum neurotoxin has been designed and evaluated. This new substrate, which contains the FRET pair Mca/Dnp was compared to the SNAPtide® substrate (Product #520 and #521) FRET peptides, which contain the oAbz/Dnp and FITC/DABCYL FRET pairs, respectively (**FIGURE 1**). The substrates were evaluated on the basis of specificity constants, k_{cat}/K_m, quenching efficiency, and the increase in relative fluorescence units (RFU) observed after complete hydrolysis (**Table 1**). Tests to estimate sensitivity with LcA were also performed (**FIGURE 2**).

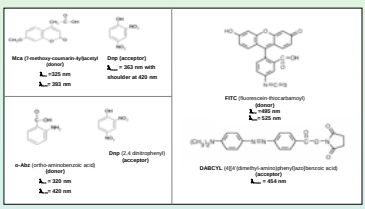

FIGURE 1: The three FRET pairs used in peptides designed as substrates for the BTA enzyme.

Fluorimetric assays: Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). Assays to determine specificity constants, k_{cat}/K_m, were performed with plates containing 10 µM FRET peptide, pre-incubated at 37ºC for 15 minutes to ensure equilibrium prior to addition of 10 nM LcA. For the sensitivity assays, plates containing serial dilutions of LcA were equilibrated for 15 min at 37ºC prior to addition of 10 µM substrate. For both assays, the time-dependent increase in fluorescence intensity was monitored at 37ºC. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz-substrate, 325 nm and 398 nm for the Mca-substrate, and 490 nm and 523 nm using a cutoff filter of 495 nm for the FITC-substrate.

Determination of specificity constants, k_{cat}/K_m: Measurements of kinetic parameters using FRET peptides may be limited by peptide solubility and the inner filter-quenching effects observed at concentrations approaching K_m. Specificity constants were determined using the progress curve method at substrate concentrations much lower than the K_m. The apparent first order rate constant was calculated by fitting the progress curves to:

$$RFU(t) = \Delta RFU[1-\exp(-k_{obs} \times t)] + RFU_o; \text{ where } \Delta RFU = RFU_{max} - RFU_o$$

The second order rate constant, k_{cat}/K_m, was calculated using:

$$k_{cat}/K_m = k_{obs}/[E_o]$$

All non-linear regressions were performed using KaleidaGraph software.

Table 1: Hydrolysis data for FRET peptides as substrates for LcA.

Peptide	k_{cat}/K_m ($M^{-1}s^{-1}$)	ΔRFU	RFU_1/RFU_o [a]	% q.e. [b]	RFU/sec
SNAPtide (oAbz/Dnp)[c]	285,330	4,970	18	94	8.62
SNAPtide (Mca/Dnp)	83,879	17,546	12	91	14.87
SNAPtide (FITC/DABCYL)[d]	22,318	30,168	11	90	7.72

[a] $RFU_1/RFU_o = RFU_{max}-RFU_{buffer}/RFU_{enzyme}-RFU_{buffer}$
[b] Quenching efficiency (%q.e.) = (1-RFU_1/RFU_o)x100
[c] List Labs SNAPtide, Product #520
[d] List Labs SNAPtide, product #521

Sensitivity: The hydrolysis of the three peptides after 2 hrs at 37ºC was evaluated as a function of LcA concentration in order to determine the minimum amount of toxin detectable using these FRET substrates. The Mca/Dnp FRET substrate is the most sensitive with a detection limit around 0.01 nM LcA or 0.5 ng/ml. The FITC/DABCYL peptide is not useful for detection of the toxin but is valuable for inhibitor screening where UV absorbing compounds could be evaluated without interference.

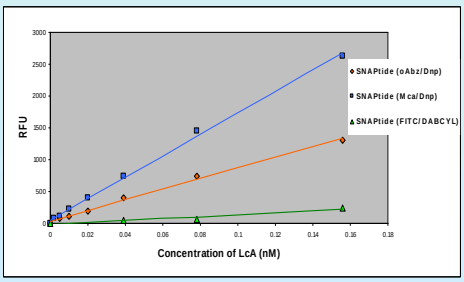

FIGURE 2: The fluorescence intensity as a function of LcA concentration for the FRET substrates after 2 hrs digestion at 37ºC. A linear relationship between the RFU and the concentration of LcA is observed.

Conclusions: The o-Abz/Dnp SNAPtide® is the best FRET peptide based on the increase in fluorescence for the fully digested peptide (RFU_1/RFU_o) and the quenching efficiency as given in **Table 1**. The best FRET substrate for the BTA enzyme, as measured by the k_{cat}/K_m, is also the oAbz/Dnp SNAPtide®. The Mca/Dnp SNAPtide® produces the strongest signal measured as RFU/sec and as such is the preferred peptide for detection of low levels of botulinum toxin (**FIGURE 2**).