



New High Affinity Antibodies Against Botulinum Neurotoxin Type A

Todd Christian, Nancy Shine, and Karen Crawford
List Biological Laboratories, Inc.
540 Division Street, Campbell, CA 95008

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.

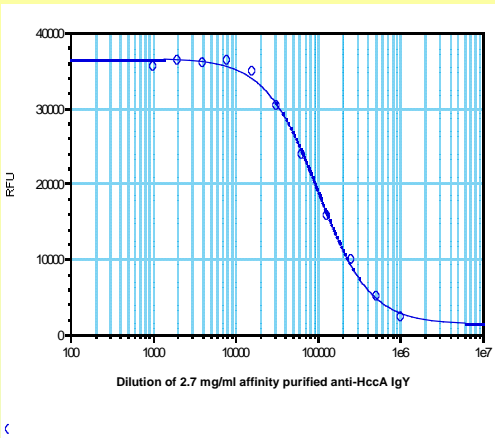


FIGURE 1: Titer determination – "Indirect" ELISA

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.

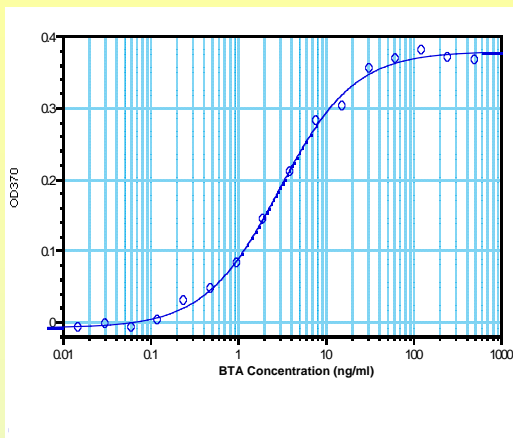


FIGURE 2: Detection of BTA – "Sandwich" ELISA

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 peroxidase 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 (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).

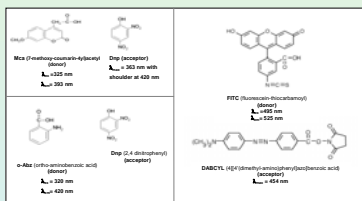


FIGURE 1: The three FRET pairs used in peptides designed as substrates for the BTA enzyme.

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 37°C 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 37°C prior to addition of 10 µM substrate. For both assays, the time-dependent increase in fluorescence intensity was monitored at 37°C. 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:

$$RFU(t) = \Delta RFU[1 - \exp(-k_{obs} \times t)] + RFU_0; \text{ where } \Delta RFU = RFU_{max} - RFU_0$$

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

$$k_{cat}/K_m = k_{obs}/[E_0]$$

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 ₀ /RFU _∞ ^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

^aRFU₀/RFU_∞ = RFU_{initial}/RFU_{final}

^bQuenching efficiency (% q.e.) = (1 - RFU₀/RFU_∞) x 100

^cList Labs SNAPtide, Product #520

^dList Labs SNAPtide, product #521

Sensitivity: The hydrolysis of the three peptides after 2 hrs at 37°C 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.

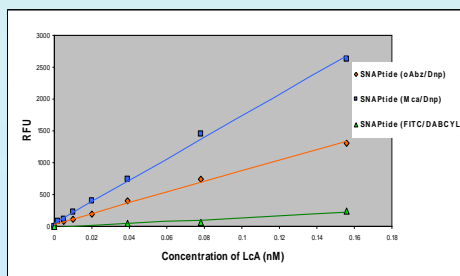


FIGURE 2: The fluorescence intensity as a function of LcA concentration for the FRET substrates after 2 hrs digestion at 37°C. A linear relationship between the RFU and the concentration of LcA is observed.

Conclusions: The o-Abz/Dnp SNAPtide® is the best FRET peptide based on the increase in fluorescence for the fully digested peptide (RFU₀/RFU_∞) 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).