

Introduction:

Botulinum neurotoxin type A (BoNT/A) development requires assays that can assess both receptor-mediated binding and catalytic activity, particularly when screening candidate toxins or formulations designed to improve potency, specificity, and stability. Two critical functional steps in BoNT/A toxicity are binding to the neuronal receptor SV2c and cleavage of the intracellular substrate SNAP25. We present a bifunctional BoNT/A assay that combines SV2c-mediated toxin capture with SNAPTide®-based detection of SNAP25-like proteolytic activity.

Purpose:

This approach enables developers to evaluate both receptor-binding integrity and enzymatic function in a single workflow and may be useful for high-throughput screening for improved BoNT/A toxins, formulations, and stability.

Background:

Botulinum toxins are synthesized as single 150 kDa polypeptide chains that are subsequently cleaved to produce a heavy chain and a light chain linked by a disulfide bond. The zinc-dependent N-terminal light chain is the catalytic subunit which, depending on the serotype of the neurotoxin, selectively cleaves one of the SNARE membrane fusion proteins. The type A neurotoxin cleaves the 25 kDa synaptosomal protein, SNAP-25, exclusively between residues Gln¹⁹⁷-Arg¹⁹⁸. The primary sequence of the C-terminal end is given below. The minimum effective BoNT/A substrate is 13 amino acids consisting of residues 190-202 of SNAP-25 (Schmidt JJ and Bostian KA, J. Protein Chem. 1997, 16:16-26). The **blue arrow** indicates the BoNT/A cleavage site.

I-D-T-Q-N¹⁷⁵-R-Q-I-D-R¹⁸⁰-I-M-E-K-A¹⁸⁵-D-S-N-K-T¹⁹⁰-R-I-D-E-A¹⁹⁵-N-Q-R-A-T²⁰⁰-K-M-L-G-S²⁰⁵-G

SNAPTide® is used as the substrate in these studies. It is based on the amino acid sequence shown above and contains the FRET pair: ortho-aminobenzoic acid (o-Abz) on the N-terminus and 2,4-dinitrophenyl (Dnp) on a lysine residue close to the C-terminus.

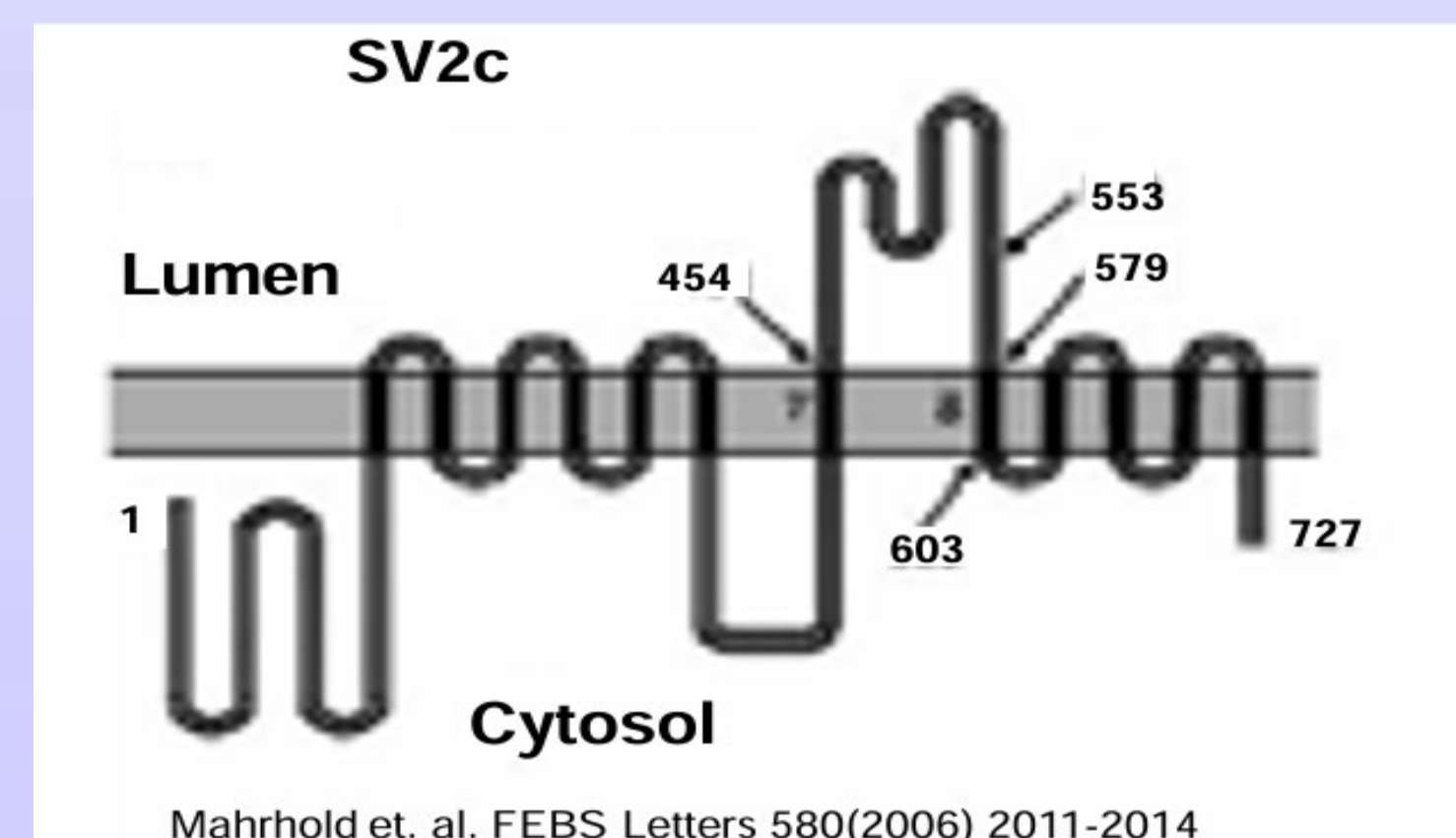


Figure 1. SV2c structure

The binding domain for Botulinum Neurotoxin Type A has been shown to be in the luminal loop, amino acids 454-579.

SV2c contains a region known as the luminal domain loop between transmembrane domains 7 and 8, and has been shown to be the location of botulinum neurotoxin type A (BoNT/A) binding. Previously we have demonstrated the functionality of the GST-SV2c fusion protein as a receptor for BoNT/A^{1,2,3}.

In this assay, the GST fusion protein containing the luminal loop of SV2c is used to specifically capture BoNT/A.

A highly sensitive version of the bifunctional assay has been presented previously. The SV2c is attached to glutathione-coated magnetic beads. The amount of SNAPTide® cleaved by BoNT/A neurotoxin is monitored using HPLC.

This assay has been optimized to allow highly sensitive detection of BoNT/A captured, on the native receptor, SV2c, and detected by BoNT/A specific enzymatic activity using as little as 1.25 pg of BoNT/A. This is significantly less than one mouse LD50 (5 pg).

SV2c-captured BoNT/A (pg)	Cleaved SNAPTide® 520			
	Response (Peak Area)	% CV	(pmoles)	(pg)
0	0	0	0	0
1.25	1053	23	0.16	14.2
2.5	1804	18	0.27	23.4
5	3670	16	0.53	46.5
10	7055	14	1.00	86.6

Table 1. Amount of cleaved SNAPTide® 520 detected in pmoles and pg after overnight exposure to various amounts of SV2c-captured BoNT/A (pg).

Background (continued)

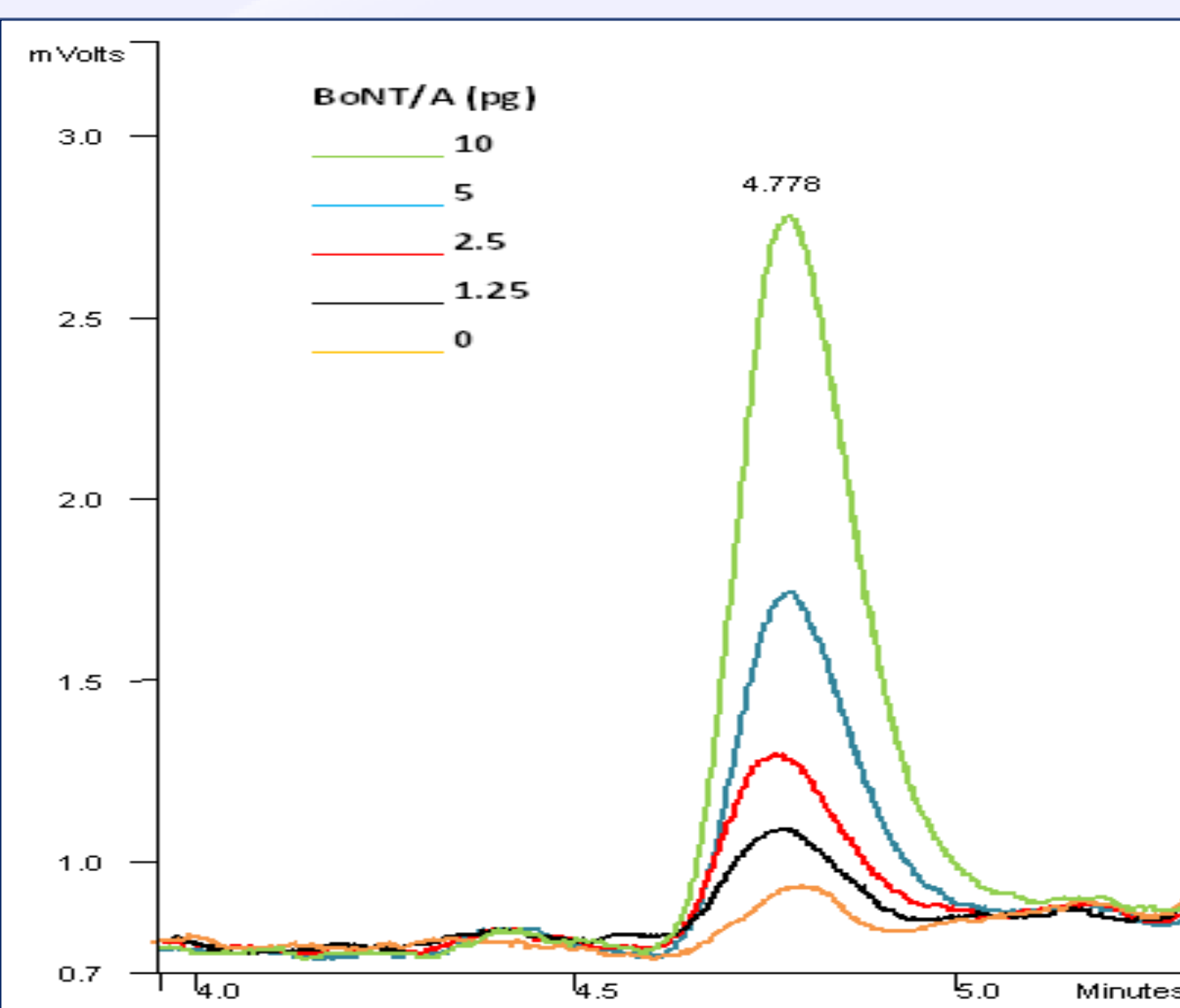


Figure 2. HPLC chromatograms of SNAPTide® 520 cleaved by SV2c captured BoNT/A. After digestion, the o-Abz-containing N-terminal fragment was visible at 4.7 min.

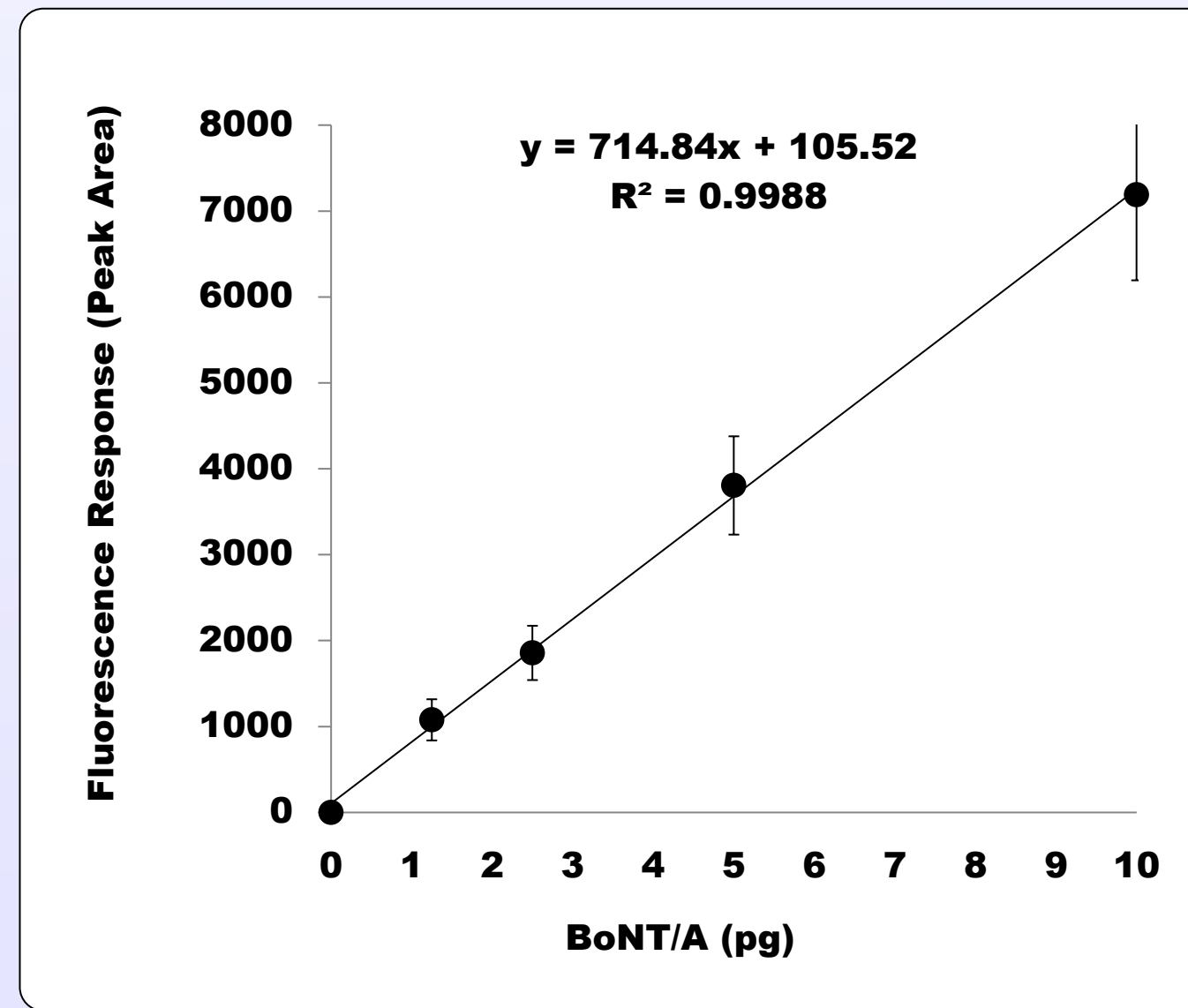


Figure 3. Plot of the peak area corresponding to the amount of cleaved SNAPTide® 520 substrate as a function of SV2c-captured BoNT/A (pg). The fluorescence response of cleaved SNAPTide® generated from SV2c-captured BoNT/A is linearly proportional to the toxin.

Materials and Methods

EXPERIMENT 1: SCREENING FOR BONT/A COMPLEX ENZYMATIC ACTIVITY.

BoNT/A complex (Prod #128) is a product of List Biological Laboratories, Inc., and has been stored long term at 2-8°C for 11 to 17 years (BoNT/A #2 LTS). The rate of cleavage of SNAPTide® by the BoNT/A complex was monitored using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) and black flat-bottomed plates (E&K Scientific, Campbell, CA). Stock solutions of the FRET substrate were made at 2.5 mM in dimethyl sulfoxide (DMSO). Final dilutions were made in the reaction buffer: 20 mM HEPES containing 10 mM ZnCl₂, 5% TWEEN-20, and 500 mM DTT. The final concentration of SNAPTide® was 10 µM. The time-dependent increase in fluorescence intensity was monitored at 37°C. The excitation wavelength was set to 320 nm and the emission to 418 nm.

EXPERIMENT 2: PLATE ASSAY TO TEST THE DUAL FUNCTION OF BOTULINUM TOXIN TYPE A COMPLEX

The goal of this assay was to evaluate the binding of BoNT/A complex to its receptor SV2c. The relative level of binding for each sample is estimated by the relative level of cleavage of SNAPTide®. Cleavage of SNAPTide® by the same amounts of BoNT/A complex free in solution was also obtained.

Four samples of BoNT/A complex were tested:

- Three aliquots of a BoNT/A complex sample from Metabio, Prod #100 (BoNT/A #1) were tested. Aliquots (~25 µg of toxin) were prepared and stored for 4 days at either 2-8°C (control), room temperature, or at 37°C before the test.
- One sample of List Prod #128 (BoNT/A #2 LTS) was also included to evaluate a BoNT/A under long term storage.

The assay consists of five steps:

Step 1: A Reacti-Bind Black Opaque Glutathione-coated 96-well plate (Pierce #15340) is washed 3 times with 200 µL/well of Tris-Buffered Saline (TBS, Thermo Fisher Scientific #28379).

Step 2: The plate is coated with GST-SV2c (List Labs Product #169). Two hundred nanograms of GST-SV2c are added to each well, and the plate is incubated at RT overnight.

Step 3: Binding of BoNT/A complex to GST-SV2c protein-coated plate. Each sample of toxin was prepared in TBS to contain 2.5 µg/100 µL buffer. The initial dilution was 2-fold to 1.25 µg/100 µL/well. Four two-fold serial dilutions of the BoNT/A complex were done. The plate was incubated for 2 hours at 37°C and then washed 4 times with TBS.

Step 4: To evaluate the BoNT/A complexes free in solution, the same dilutions of the complexes were added to wells that were not coated with SV2c.

Step 5: Cleavage of SNAPTide® by BoNT/A complex. Stock solutions (5 mM) of the SNAPTide® (List Labs Product #520) FRET peptide were made in dimethyl sulfoxide (DMSO). The SNAPTide® was diluted in 20 mM HEPES, pH 8.0, containing 1.0 mM ZnCl₂, 5 mM DTT, and 0.1% Tween-20 (reaction buffer). SNAPTide® in reaction buffer was added to the BoNT/A complexes bound and free. The plate was placed in the microplate reader at 37°C for 3 hours. The rate of cleavage of the SNAPTide® was monitored (RFU/min), followed by an endpoint read. The plate was then removed from the plate reader and left at room temperature overnight. Final endpoint readings were obtained after overnight Day 1, 2, 3, and 4.

References

- Suryadi, K*, Christian, T., and Shine, N., Sensitive And Specific Bifunctional Assay For Botulinum Neurotoxin Type A, Presented at the 49th Annual Interagency Botulinum Research Coordinating Committee Meeting, September 2012 in Baltimore, MD
- T. Christian, K. Suryadi and N. Shine. New SV2c Construct for Use in Binding and Detecting Botulinum Neurotoxin Type Presented at the 7th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins, October 2011 in Santa Fe, NM, USA.
- T. Christian and N. Shine. Capture Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c. The 6th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins, June 2008 in Bavaria, Italy.
- T. Christian, A. Rummel, and N. Shine. Functional Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c. The 45th Annual Interagency Botulinum Research Coordinating Committee Meeting, September 2008 in Philadelphia, PA.
- T. Christian and N. Shine. Sensitive, In Vitro, Bifunctional, Potency Assay For Botulinum Neurotoxin Type A. The 46th Annual Interagency Botulinum Research Coordinating Committee Meeting, October 2009 in Alexandria, VA.

Results

EXPERIMENT 1: SCREENING FOR BONT/A #2 LTS ENZYMATIC ACTIVITY

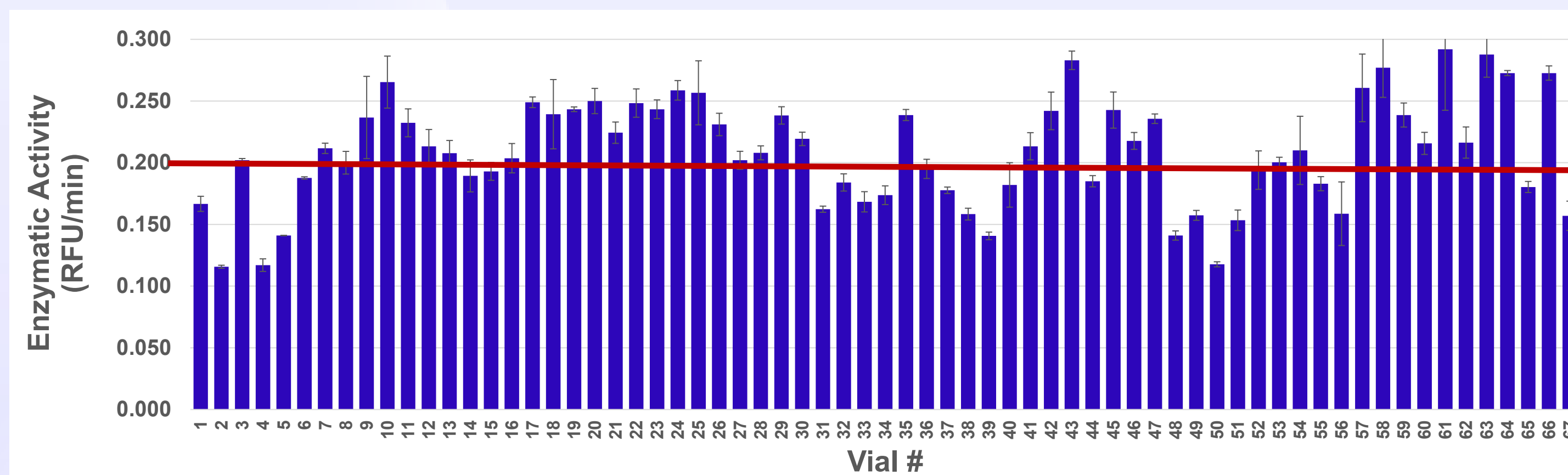
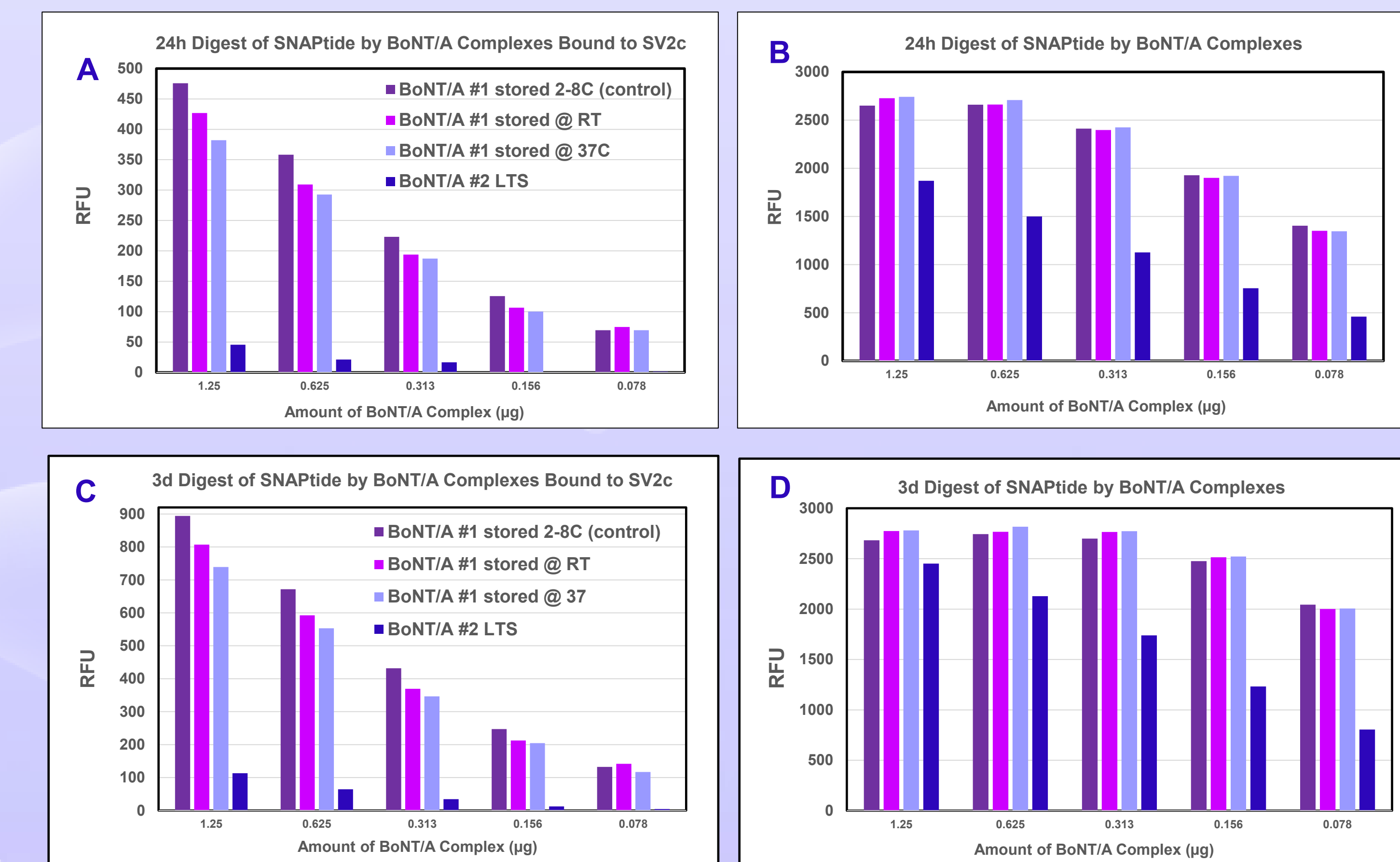


Figure 4. Plot of the rate of cleavage of SNAPTide® by BoNT/A #2 LTS. The screen covers vials that have been stored for 11-17 years, left to right, respectively, at 2-8°C. The vials contain 10 µg of BoNT/A complex, and the expected rate of cleavage is ≥ 0.2 RFU/min, as indicated by the horizontal line. Data are the average of 3 replicates, and standard deviations are indicated. Twenty of the 67 vials had less activity (<0.2 RFU/min) than expected.

EXPERIMENT 2: PLATE ASSAY TO TEST THE DUAL FUNCTION OF BoNT/A #1 and BoNT/A #2 LTS COMPLEXES

Figure 5. Endpoint readings after 24 hour and 3 day cleavage of SNAPTide® by BoNT/A complexes bound to SV2c (A, C) and free in solution (B, D)



Conclusions

As shown in **Figure 4**, SNAPTide® detected measurable differences in the enzymatic activity of a lot of BoNT/A complex (BoNT/A #2 LTS) at 2-8°C under long-term storage. Although loss in enzymatic activity did not correlate to storage duration, it is suspected that vials were impacted by variable storage conditions due to defective closure. SNAPTide® can be used as a quick and easy screen to screen for enzymatic activity.

As shown in **Figure 5**, the bifunctional assay of BoNT/A toxicity can be used to evaluate both the integrity of the heavy chain SV2c protein receptor site as well as the enzymatic activity associated with the BoNT/A light chain. The bar graphs shown for the BoNT/A complexes after 24 hours and 3 days (**Figures 5 A, C**) clearly indicate that the binding of SV2c is impacted by the storage temperature. The level of bound BoNT/A #1 is the greatest for the control stored at 2-8°C; the level is decreased for the sample stored at RT, and the sample stored at 37°C has the lowest amount of bound complex. The binding of BoNT/A #2 LTS to SV2c is minimal. After 3 days (**Figure 5 C**), the BoNT/A complexes bound to SV2c continue to digest the SNAPTide® at the same relative levels as seen after 24 hours.

The enzymatic activity of the same amounts of BoNT/A complexes free in solution is shown in **Figures 5B and D** after incubation for 24 hours and 3 Days, respectively, indicating total digestion of the SNAPTide® for the two higher amounts of BoNT/A #1 after storage at 2-8°C and RT. After 3 days, the BoNT/A #1 held at 37 °C has also digested all of the SNAPTide®. Interestingly, the BoNT/A #2 LTS retains a significant amount of enzymatic activity, while the binding to SV2c has been impacted by the long-term storage.

The SV2c/SNAPTide® bifunctional assay provides a practical workflow for evaluating two key functional attributes of BoNT/A: receptor binding and SNAP25-like catalytic activity. By detecting changes in both binding integrity and enzymatic function, the assay supports screening for BoNT/A candidates and formulation that preserve both receptor binding and catalytic activity for improved potency, specificity, and stability.